Multifunctional approach of L-Dopa induced dyskinesia pathophysiology in Parkinson's disease : from the striatum to the whole brain

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THÈSE

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Approche expérimentale de la physiopathologie des dyskinésies L-Dopa induites dans la maladie de Parkinson :

Comparaison de la cible classique, le striatum avec l'ensemble du cerveau.

Membres du Jury

M. Jean-Antoine Girault.....Président
Mme. Jocelyne Caboche .....Rapporteur
M. Emmanuel Valjent .....Rapporteur
M. Erwan Bézard.....Examinateur
M. Christian E. Gross.....Directeur de thèse

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THESIS

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Jury members

Mr Jean-Antoine Girault .....President
Mrs Jocelyne Caboche .....External examiner
Mr Emmanuel Valjent.....External examiner
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Résumé

Le traitement de référence de la maladie de Parkinson (MP) reste l'utilisation du précurseur direct de la dopamine: la L-3,4-dihydroxyphenylalanine (L-Dopa). Le traitement chronique des patients parkinsoniens à la L-Dopa induit, en revanche, systématiquement des mouvements involontaires anormaux que l'on qualifie de dyskinésies induites par la L-Dopa (DIL). L'étude de l'expression des dyskinésies s'est essentiellement focalisée sur les dysfonctions neuronales engendrées dans les régions motrices des ganglions de la base et a permis de révéler une surexpression significative de gènes de réponse précoce (GRP) tels que: ΔFosB, ARC, Zif268 et FRA2 dans le striatum de rats dyskinétiques traités chroniquement à la L–Dopa.

En revanche, plusieurs autres régions dopaminoceptives, probablement affectées par la dopamine exogène nouvellement synthétisée, ont été négligées alors qu'elles pourraient jouer un rôle clé dans l'expression des dyskinésies. Par conséquent, nous avons quantifié l'expression de ΔFosB, ARC, FRA2 et Zif268 dans l'ensemble du cerveau de rats dyskinétiques que nous avons comparée à des rats non-dyskinétiques. Cette approche nous a permis d'identifier 9 structures, localisées en dehors des ganglions de la base, présentant une surexpression d'au moins 3 des GRPs cités ci-dessus. Parmi ces structures, le domaine dorsolatéral du « bed nucleus of the stria terminalis » (dlBST) et l'habenula latérale (LHb) montrent une corrélation significative entre l'expression de ΔFosB et la sévérité des dyskinésies. Nous avons donc fait l'hypothèse que ces 2 structures pouvaient être impliquées dans l'expression des dyskinésies. Par conséquent, pour évaluer le rôle potentiel du dlBST et de la LHb dans les dyskinésies, nous avons inhibé l'activité électrique des neurones exprimant FosB/ΔFosB en utilisant la méthode d'inactivation sélective du Daun02/ß-galactosidase que nous avons précédemment validée dans une structure bien connue pour être impliquée dans les dyskinésies: le striatum. Nous avons démontré que l'inhibition de ces neurones, à la fois dans le dlBST et la LHb, diminuait la sévérité des dyskinésies sans affecter l'effet bénéfique de la L-Dopa chez les rats dyskinétiques. Nous avons ensuite pu confirmer l'implication du dlBST grâce au model de référence des dyskinésies: le macaque dyskinétique lésé au MPTP. L'ensemble de ces résultats nous a ainsi permis de montrer, pour la première fois, l'implication fonctionnelle de 2 structures externes aux ganglions de la base dans l'expression des dyskinésies, offrant de nouvelles perspectives thérapeutiques.

Mots clés: Maladie de Parkinson; Dyskinésies induites par la L-Dopa; gènes de réponse précoce; stéréologie; 2-deoxyglucose; électrophysiologie; daun02; rats; macaques.

Abstract

The gold standard treatment for Parkinson's disease (PD) remains the dopamine precursor L-3,4-dihydroxyphenylalanine (L-Dopa). Long-term L-Dopa treatment systematically leads to abnormal involuntary movements (AIMs) called L-Dopa-induced dyskinesia (LID). These manifestations first led to investigate the neuronal dysfunctions in the motor regions of the basal ganglia and unravelled an overexpression of ΔFosB, ARC, Zif268 and FRA2 immediate-early genes (IEG) in the dopamine-depleted striatum of dyskinetic rats.

However, other several dopaminoceptive structures, likely affected by the exogenously produced dopamine, have been neglected although they might play a key role in mediating LID.

Hence, we assessed the expression of ΔFosB, ARC, FRA2 and Zif268 IEGs in the whole brain of dyskinetic rats compared to non-dyskinetic ones. Such approach shed light notably upon 9 structures located outside of the basal ganglia displaying an IEG overexpression. Among them, the dorsolateral bed nucleus of the stria terminalis (dlBST) and the lateral habenula (LHb) displayed a significant correlation between ΔFosB expression and LID severity. We therefore postulated that these structures might play a role in LID manifestation.

Therefore, to assess dlBST and LHb causal roles upon LID severity, we inhibited the electrical activity of FosB/ΔFosB-expressing neurons using the selective Daun02/β-galactosidase inactivation method that we previously validated in a well known structure involve in LID: the striatum. Interestingly, the inactivation of dlBST and LHb ΔFosB-expressing neurons alleviated LID severity and increased the beneficial effect of L-Dopa in dyskinetic rats. Remarkably, BST involvement in LID was confirmed in the gold standard model of LID, the dyskinetic MPTP-lesioned macaque. Altogether, our results highlight for the first time the functional involvement of 2 structures outside of the basal ganglia in LID, offering new targets to innovative treatments of LID.

Keywords: Parkinson's disease; L-Dopa induced dyskinesia; immediate early genes; stereology; 2-deoxyglucose; electrophysiology; daun02; rats; macaques

Résumé substantiel

La maladie de Parkinson est une maladie neurodégénérative caractérisée par une perte progressive de plusieurs populations neuronales incluant notamment les neurones dopaminergiques de la substance noire *pars compacta*. Sur le plan clinique, cette maladie se traduit par 4 symptômes moteurs majeurs : l'akinésie, la rigidité articulaire, l'instabilité posturale et les tremblements. L'objectif des traitements actuels est de pallier la déficience en dopamine soit par l'utilisation d'agonistes dopaminergiques, soit par l'administration de Levodopa (L-Dopa), un précurseur direct de la dopamine capable de passer la barrière hémato-encéphalique contrairement à cette dernière. Bien qu'efficace pendant quelques années, le traitement à la L-Dopa induit systématiquement des complications motrices se traduisant par des mouvements anormaux involontaires appelés dyskinésies induites par la L-Dopa (DIL).

A l'heure actuelle, il n'existe pas de traitement efficace permettant de lutter contre les dyskinésies. Néanmoins, plusieurs stratégies sont utilisées afin de soulager les patients. Tout d'abord, afin de retarder au maximum la prise de L-Dopa, des agonistes dopaminergiques peuvent être administrés seuls ou en combinaison avec la L-Dopa au stade initiale de la maladie. Il est également possible de stabiliser les taux de dopamine dans le cerveau en administrant des inhibiteurs des enzymes de dégradation de la dopamine comme la cathécol-O-méthyl-transférase (tolcapone, entacapone) ou la monoamine oxydase B (sélégiline, rasagiline). Au niveau des traitements pharmacologiques anti-dyskinétiques, seul l'Amantadine est utilisée. Cependant elle reste à usage limité au vu de son efficacité et des effets secondaires indésirables engendrés. Sur le plan neurochirurgical, une intervention est également possible. Son objectif est de permettre une stimulation cérébrale profonde soit du noyau sous-thalamique, soit du globus pallidus interne. Cette approche permet non seulement de diminuer les dyskinésies mais également de réduire de moitié les doses de L-Dopa administrées aux patients.

Bien qu'au cours de ces dernières années l'évolution des connaissances sur les dyskinésies a permis de mettre en évidence de nouveaux concepts, ces derniers se sont concentrés uniquement sur un groupe de structures appelés ganglions de la base composés du striatum, du globus pallidus externe (GPe) et interne (GPi), de la substance noire et du noyau sous-

thalamique. Malgré les connaissances accumulées sur la physiopathologie des dyskinésies associées à ces structures, il n'existe, à ce jour, aucune certitude sur les mécanismes qui sous-tendent les dyskinésies. Les ganglions de la base, bien qu'ayant un impact central dans la maladie de Parkinson et dans les dyskinésies, ne sont pas des structures isolées et échangent des informations avec un grand nombre de régions cérébrales motrices ou non-motrices. En effet, plusieurs autres régions dopaminoceptives, probablement affectées par la dopamine exogène nouvellement synthétisée suite à l'administration de L-Dopa, ont été négligées alors qu'elles pourraient jouer un rôle clé dans l'expression des dyskinésies. Ainsi l'objectif de ma thèse a consisté à identifier si des structures situées en dehors des ganglions de la base pourraient être impliquées dans l'expression des dyskinésies.

Afin de répondre à cet objectif, nous avons, dans une première étude, quantifié l'expression de gène de réponse précoce (GRP) dans l'ensemble du cerveau. Les GRP sont une classe particulière de gènes très rapidement transcrits suite à un stimulus externe permettant ainsi d'identifier une réponse génomique induite par un événement extérieur, comme les DIL. Nous avons choisis de quantifier l'expression de 4 GRP : ΔFosB, ARC, Zif268 et FRA2. Le choix de ces GRP s'est basé sur des données de la littérature démontrant notamment une augmentation de l'expression de ces gènes dans le striatum de rats préalablement déplétés en dopamine puis traités avec des composés dopamimétiques. Bien qu'il serait tentant de corréler l'expression des GRP à l'activité électrophysiologique d'une structure cérébrale, nous devons garder à l'esprit que cette relation, souvent considérée comme acquise, n'est actuellement pas démontrée, tout du moins pour les GRP sélectionnés dans cette étude. Par conséquent, l'expression des GRP doit être considérée uniquement comme un marqueur de l'activité transcriptionnel et non comme un marqueur de l'activité électrophysiologique tant que cette relation n'a pas été démontrée. Dans cette étude, la dose de L-Dopa utilisée a été ajusté juste en dessous de son EC50 (i.e. 3,2mg/kg) afin d'obtenir 2 populations de rats : des rats présentant des dyskinésies qualifiés de dyskinétiques et des rats ne présentant pas de problèmes moteurs, qualifiés de non-dyskinétiques. Par conséquent, la quantification de l'expression des 4 GRP dans le cerveau entier de rats dyskinétiques par rapport à des rats non-dyskinétiques nous a permis d'identifier des régions cérébrales présentant une réponse transcriptionnelle induite par l'expression des dyskinésies.

Ainsi, cette étude nous a permis de confirmer la surexpression de ΔFosB, ARC, Zif268 et FRA2 dans les structures classiquement étudiées dans les dyskinésies comme le striatum, la substance noir par compacta (SNc) et le cortex moteur M1. En revanche, aucune expression

n'a pu être détectée dans le GPe et le noyau sous-thalamique (NST). En dehors des ganglions de la base, nous avons pu identifier 9 structures présentant une surexpression d'au moins 3 GRP chez des rats dyskinétiques tels que la région dorsal (dlBST) (composé du noyaux oval (ovBST) et juxta (jxBST)) et la région médiale (mBST) du noyaux du lit de la strie terminale (BST), la région rostrale de la zona incerta (rZI), l'habenula latéral (LHb), l'Hippocampe, les noyaux pontins (Pn), le noyau cunéiforme (CnF) et le noyau pédonculopontin (PTg). Puis, afin de renforcer le lien entre l'expression des GRP et des DIL, nous avons corrélé le nombre de cellules immuno-positives pour les GRP avec la sévérité des dyskinésies. Cette analyse nous a permis de confirmer les données de la littérature en démontrant une corrélation significative entre les cellules marquées pour DFosB dans le striatum et la sévérité des dyskinésies bien que notre étude est la première à le démontrer en réalisant une quantification par la méthode de stéréologie non biaisée. Puis, nous avons également montré des corrélations significatives pour des structures en dehors des ganglions de la base. Premièrement, les 2 noyaux du dlBST ont montré des corrélations significatives entre l'intensité des dyskinésies et, respectivement, le nombre de cellules marquées pour DFosB dans l'ovBST et FRA2 dans le jxBST. Au niveau de l'épithalamus, la LHb a montré une corrélation significative entre l'intensité des dyskinésies et le nombre de cellules marquées pour ARC et DFosB. Enfin, au niveau du tronc cérébral, le Pn et CnF ont montré des corrélations significatives entre l'intensité des dyskinésies et, respectivement, le nombre de cellules marquées pour Zif268 et FRA2. Par conséquent, cette première étude nous a permis de démontrer que les domaines à la fois moteurs et non-moteurs des boucles cortico-sous-corticales présentaient des corrélations significatives entre le nombre de cellules marquées pour DFosB, ARC, Zif268 et FRA2 et la sévérité des dyskinésies.

Nous nous sommes ensuite intéressés aux propriétés des structures précédemment identifiées suite à l'expression des dyskinésies. Ainsi, nous avons montré une augmentation de la potentialisation à long terme des courants inhibiteurs post-synaptiques $GABA_A$ engendrée par les récepteurs dopaminergiques D1 dans l'ovBST de rats dyskinétiques. Puis, nous avons démontré que la LHb présentait une diminution d'accumulation de 2-déoxyglucose (2-DG) chez des macaques dyskinétiques en comparaison avec des macaques non-dyskinétiques, parkinsoniens et sham. De plus, nous avons montré que les patterns ainsi que l'activité de décharge des neurones de la LHb étaient modifiés de manière significative suite à un traitement chronique à la L-Dopa chez des rats dyskinétiques. Ainsi, l'ensemble de ces résultats confirme que l'activité neuronale du BST et de la LHb, altérée en réponse à

l'administration de L-Dopa, est liée à l'expression des dyskinésies. Nous nous sommes donc concentrés sur ces 2 noyaux dans la suite de nos travaux.

La démonstration du rôle spécifique de ces structures externes aux ganglions de la base dans la physiopathologie des dyskinésies a nécessité une modulation sélective de leur activité électrophysiologique puis d'évaluer son impact sur la sévérité des dyskinésies. Ainsi, afin d'évaluer le rôle causal des structures précédemment identifiées dans la physiopathologie des dyskinésies, nous avons utilisé la méthode d'inactivation sélective du Daun02/b-galactosidase. La méthode du Daun02 a été originellement développée dans le traitement des cancers. Cette méthode consiste en l'administration locale d'une pro-drogue : le Daun02 qui est converti en Daunorubicine par la b-galactosidase exprimée dans les cellules de mammifères par la transduction préalable du gène *LacZ* sous le contrôle d'un promoteur cellule-spécifique. Il a été montré que la Daunorubicine était capable de diminuer l'activité électrique de neuroblastome. La méthode du Daun02 a été apportée aux neurosciences par le groupe de Bruce Hope dans le domaine de l'addiction et a été précédemment utilisée dans le cortex préfrontal et le nucleus accumbens. De manière surprenante, aucune de ces études n'a démontré que la Daunorubicine réprimait correctement l'excitabilité neuronale suite à l'injection de Daun02. Par conséquent, avant d'utiliser cette technique dans le BST et la LHb, nous devions la valider à la fois sur le plan électrophysiologique et sur le plan comportemental dans une structure connue pour être impliquée dans les dyskinésies. De manière évidente, le striatum est apparu comme le choix parfait tant cette structure est centrale dans la physiopathologie des dyskinésies.

Ainsi, nous avons commencé par démontrer qu'à la fois, l'application de Daun02 sur des cultures de neurones striataux de rats exprimant constitutivement la b-galactosidase et l'application directe de Daunorubicine sur des tranches striatales de cerveau de rats étaient capables de diminuer significativement l'excitabilité des neurones striataux sans affecter leur viabilité. Par conséquent, cette étude nous a permis de valider la méthode du Daun02 sur le plan électrophysiologique dans des neurones striataux, nous donnant ainsi l'opportunité de tester cette méthode *in vivo*.

Parmi les altérations moléculaires associées aux dyskinésies, l'accumulation de DFosB a été identifiée dans le striatum comme un marqueur de cette pathologie à la fois chez les rongeurs, les primates non-humains et les humains. De plus, plusieurs études indiquent que DFosB est

largement impliqué dans l'expression de comportement à long terme associé à la stimulation du système dopaminergique. Ainsi, la répression de DFosB par interférence moléculaire chez les rongeurs ou par la surexpression de DJunD (le dominant négatif de DFosB) chez les primates non-humains a permis de diminuer à la fois le commencement et l'expression des dyskinésies. Par conséquent, DFosB n'est pas seulement un marqueur des dyskinésies mais a également un impact fonctionnel sur l'expression des dyskinésies. L'ensemble de ces données nous a donc amené à exprimer la ß-galactosidase sous le contrôle d'un promoteur FosB que nous avons inséré dans un vecteur lentiviral pour inactiver sélectivement les neurones exprimant FosB/ΔFosB suite à l'injection de Daun02 chez des modèles animaux parkinsoniens et dyskinétiques. Nous avons démontré que l'inhibition de l'activité électrique des neurones striataux exprimant FosB/ΔFosB, induite par l'injection de Daun02, permet de diminuer la sévérité des dyskinésies à la fois chez les rats et les primates non-humains sans affecter l'effet bénéfique de la L-Dopa. Par conséquent, cette étude démontre, pour la première fois, le lien causal entre l'activité électrique des neurones striataux exprimant FosB/ΔFosB et la sévérité des dyskinésies.

Après avoir démontré que la méthode du Daun02 permettait de soulager les dyskinésies, nous avons utilisé cette technique pour évaluer le rôle du dlBST et de la LHb dans l'expression des dyskinésies en inactivant les neurones exprimant FosB/ΔFosB de ces structures. Nous avons établi que l'inactivation de ces neurones à la fois dans le dlBST et la LHb de rats dyskinétiques permettait de diminuer la sévérité des dyskinésies tandis que l'effet antiparkinsonien de la L-Dopa était augmenté uniquement suite à l'inactivation des neurones de la LHb. Puis, nous avons pu confirmer de manière remarquable l'implication du dlBST chez des macaques dyskinétiques par une diminution de la sévérité des dyskinésies sans affecter l'effet bénéfique de la L-Dopa suite à l'injection de Daun02 dans cette structure. Par conséquent, l'ensemble de nos résultats démontre, pour la première fois, l'implication fonctionnelle de 2 structures externes aux ganglions de la base dans la physiopathologie des dyskinésies, offrant ainsi de nouvelles opportunités thérapeutiques.

Au cours de mon doctorat, notre travail a permis de mettre en lumière les altérations globales induites par un traitement chronique à la L-Dopa dans la maladie de Parkinson. Nous avons démontré que ce traitement n'impacte pas uniquement les structures classiquement étudiées dans la physiopathologie des dyskinésies mais l'ensemble du cerveau via des modifications moléculaires induisant des altérations dans la plasticité synaptique mettant en œuvre,

notamment, les GRP. Il est intéressant de noter que ces modifications impliquent des circuits moteurs, cognitifs et limbiques à la fois à l'intérieur et à l'extérieur des ganglions de la base. Par conséquent, nous proposons que l'impact fonctionnel des neurones exprimant les GRP sous-tende, entres autres, des mécanismes neuronaux impliqués dans la physiopathologie des dyskinésies induisant des complications motrices qui pourraient être amplifiées directement ou indirectement par des composantes affectives, motivationnelles et cognitives induites par un traitement chronique à la L-Dopa.

La prise en considération des mécanismes impliquant à la fois les altérations motrices et non-motrices pourrait fournir une vue plus intégrative de la physiopathologie des dyskinésies. En effet, les comportements ne sont pas uniquement liés à la motricité mais ils incluent également une composante motivationnelle : « je me déplace pour prendre un verre car j'ai soif ». Par conséquent, les altérations pathologiques entrainées par un traitement chronique à la L-Dopa dans des structures externes aux ganglions de la base devraient être étudiées plus en détails pour fournir une meilleurs compréhension des composantes multifactorielles impactant les complications motrices engendrées par les dyskinésies.

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Abbreviation list

Behavioural, cellular and molecular components

5-HT : Serotonin

6-OHDA : 6-hydroxydopamine

AIMs : Abnormal involuntary movements

AMPA : α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

NMDA : N-methyl-D-aspartate receptor

DA : Dopamine

DAergic : Dopaminergic

DDS : Dopamine dysregulation syndrome

DBS : Deep brain stimulation

DRT : Dopamine Replacement Therapy

DR : Dopamine receptor

GABA : γ-aminobutyric acid

GPCR : G protein-coupled receptors

GRK : G protein-coupled receptor kinases

ICD : Impulse control disorders

IEG : Immediate early gene

L-Dopa : L-$_{3,4}$-dihydroxypheylalanine

LID : L-Dopa induced dyskinesia

LTP : Long term potentiation

LTD : Long term depression

MPTP : 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSN : Medium spiny neurons

N/OFQ : Nociceptin/orphanin FQ

PD : Parkinson's disease

SSRIs : Selective serotonin re-uptake inhibitors

UPDRS : Unified Parkinson's Disease Rating Scale

Brain structures:

dlBST : Dorsolateral bed nucleus of the stria terminalis

CnF : Cuneiform nucleus

GPe : External segment of the globus pallidus

GPi : Internal segment of the globus pallidus

HIPP : Hippocampus

jxBST : juxta nucleus of the dorsolateral bed nucleus of the stria terminalis

LHb : Lateral Habenula

mBST : medial bed nucleus of the stria terminalis

MFB : medial forebrain bundle

ovBST : oval nucleus of the dorsolateral bed nucleus of the stria terminalis

PFC : Prefrontal cortex

Pn : Pontine nuclei

PTg : Pedunculopontine tegmental nucleus

rZi : Rostral zona incerta

SNc : Substantia nigra pars compacta

SNr : Substantia nigra pars reticulata

STN : Subthalamic Nucleus

VTA : Ventral Tegmental Area

Foreword and project aims

Parkinson's disease (PD) is a neurodegenerative disease mainly characterized by the progressive loss of dopaminergic neurons in the substantia nigra *pars compacta*. Dopamine (DA) replacement by L-Dopa administration, the direct dopamine precursor, remains the most effective form of oral symptomatic treatment for motor parkinsonian symptoms. However, 3 to 5 years after the first L-Dopa administration, most of the patients treated with L-Dopa experience debilitating side effects, mainly characterized by abnormal involuntary movements called L-Dopa induced dyskinesia. (LID).

My PhD experimental work belongs to the thematic of the research team "Pathophysiology of parkinsonian syndromes" headed by Erwan Bezard, studying the cellular and molecular mechanisms underlying movement alterations in PD and LID. Our experimental studies are conducted on the basis of pathophysiological investigations with a translational approach from cell to small and large animal models in order to identify putative therapeutic targets.

I will introduce my PhD manuscript by a review, commissioned by Progress in Neurobiology, focussing on LID pathophysiology. We organized this review by first presenting the LID clinical specifics and treatments followed by the description of currently used animal models and by reviewing the fundamental research on LID pathophysiology.
In addition, this review aims at focusing on changes specifically observed at the peak of dose of L-Dopa action. Indeed, in the literature, LID pathophysiology refers to various states. In several papers, animals are considered as “dyskinetic” (i.e. since they have been chronically exposed to L-Dopa) but they were terminated OFF L-Dopa (i.e. more than 3 hours after their last L-Dopa injection). While the OFF state is very interesting and informative on the neuronal plasticity induced by the chronic treatment, it could not be considered as the ON LID state. Indeed, the ON LID state reflects the neuronal pathological events occurring at the peak of dose of the treatment, at which dyskinesia are the most strongly expressed, and allows a correlation between the progressive L-Dopa induced motor response and the cellular alterations. We will therefore structure the introductory review by clearly distinguishing:

- Naïve animals: never exposed to dopamimetics
- The ON state: peak of dose of L-Dopa, with or without LID
- The OFF state: animals otherwise dyskinetic when challenged.

This introductory review highlights that basic and clinical research on LID have focused essentially on the basal ganglia motor circuits, undoubtedly central in LID pathophysiology. Consequently, as the main target of nigral DA neurons, the striatum has received most attention to understand the pathophysiology of LID. However, several other dopaminoceptive structures, outside of the basal ganglia and likely affected by the exogenously produced dopamine, have been neglected although they might play a key role in mediating LID. Hence, we hypothesized that structures outside of the basal ganglia could be involved in LID.

This hypothesis leads to the objective of my PhD, which aimed to identify if structures outside of the basal ganglia could potentially be affected by a chronic L-Dopa treatment.

To test this hypothesis, we first developed an unbiased screening of immediate-early gene (IEG) expression in the whole brain of dyskinetic and non-dyskinetic rats to identify putative structures differentially modified following LID expression. Then, to assess the casual role of the identified brain nuclei on LID severity, we used a selective inactivation method to decrease the neuronal excitability of a specific neuronal population in a given structure. Our results highlighted, for the first time, the functional involvement of 2 structures outside of the basal ganglia in LID, offering new putative targets to innovative treatments.

My PhD manuscript is divided in four parts:

- The introduction, containing a review on LID pathophysiology focusing on the clinical and fundamental actual knowledge of LID.
- The results, presenting 2 published, 2 submitted and 1 draft publications in the context of this PhD.
- The discussion, in which we tried to learn from our investigations to consider and understand both the role and impact of structures outside of the basal ganglia in the pathophysiology of LID.
- Supplementary publication: 1 published.

Introductory Review

Pathophysiology of L-dopa-induced dyskinesia in Parkinson's disease

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1. Abstract

Involuntary movements, or dyskinesias, represent a debilitating complication of levodopa therapy for Parkinson's disease. Dyskinesia is, ultimately, experienced by the vast majority of patients. The present review attempts to provide an overview of the current understanding of dyskinesia pathophysiology, a field that has dramatically evolved in the past twenty years. Facing the booming of data and research directions, we felt pivotal to frame the concepts, highlight the most suited models, review the myriad of data involving the striatum as well as several other brain structures, and propose a pathophysiological framework. This review has the goal to advance in our understanding of LID specifically as they might relate to the development of novel therapeutic strategies aimed to prevent the generation of dyskinetic symptoms

2. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is observed in approximately 1% of the population over 55, the mean age at which the disease is first diagnosed. PD was first described by James Parkinson (Parkinson, 1817) and consists of a syndrome including bradykinesia/akinesia, rigidity, postural abnormalities and tremor. The principal pathological characteristic of PD is the progressive death of the pigmented neurons of the Substantia Nigra pars compacta (SNc) (Hassler, 1938). The discovery, in 1960, that degeneration of the dopamine (DA) supplying neurons of the SNc causes parkinsonism (Ehringer and Hornykiewicz, 1960) opened the way for the development of pharmaceutical therapies for PD that act to enhance synaptic DA transmission using the DA precursor L-$_{3,4}$-dihydroxypheylalanine (L-Dopa) (Birkmayer and Hornykiewicz, 1961, 1962; Lees, 1994; Yahr *et al.*, 1968).

The initial exuberance surrounding the positive effects of L-Dopa in PD soon gave way to the recognition that long-term L-Dopa therapy is confounded by the development of adverse events related to fluctuations in motor response. These motor fluctuations are changes in the quality of motor response following long-term treatment with L-Dopa. Motor fluctuations include on-off fluctuations, sudden, unpredictable changes in mobility, and the wearing-off phenomenon, a decrease in the duration of action of L-Dopa. However, the most debilitating

class of motor fluctuation is involuntary movements known as L-Dopa-induced dyskinesia (LID). These abnormal involuntary movements (AIMs) were first reviewed in 1974 by Duvoisin who found that after 6 months of treatment over half of patients had developed dyskinesia (Duvoisin, 1974). Ultimately, the majority of L-Dopa-treated patients experience dyskinesia, with up to 80% of patients having dyskinesia within 5 years of treatment (DeJong *et al.*, 1987; Lees and Stern, 1983; Lesser *et al.*, 1979; Marsden *et al.*, 1982; Rajput *et al.*, 1984). It should be noted that treatment-related dyskinesia are not solely a problem of L-Dopa and that DA receptor agonists are also capable of eliciting dyskinesia and within the context of this review, the commonly-used term, LID will be used, as it is widely understood, to describe dopaminergic (DAergic) treatment–related dyskinesia generally. In the past twenty years, the understanding of the neural mechanisms underlying LID in PD strongly advanced (Bezard *et al.*, 2001b; Cenci *et al.*, 1998; Fasano *et al.*, 2010; Fieblinger *et al.*, 2014; Fisone and Bezard, 2011; Jenner, 2008). Dyskinesia has been associated with a sequence of events that include pulsatile stimulation of DA receptors, downstream changes in proteins and genes, abnormalities in non-DAergic transmitter systems all of which combine to produce alterations in the neuronal firing patterns that signal between the basal ganglia and the cortex (**Figure 1**).

In this review, we aim at focusing on changes affecting both DAergic and non-DAergic transmission, and more particularly at changes observed at the peak of dose of L-Dopa, that is when dyskinesias are more expressed - ON dyskinesia - by opposition to the OFF dyskinesia situation in primed individuals or animals that has also been investigated. We also review the number of other L-Dopa-induced side-effects with an highlight of their pathophysiology when documented. This article has the goal to advance in our understanding of LID specifically as they might relate to the development of novel therapeutic strategies aimed to prevent the generation of dyskinetic symptoms.

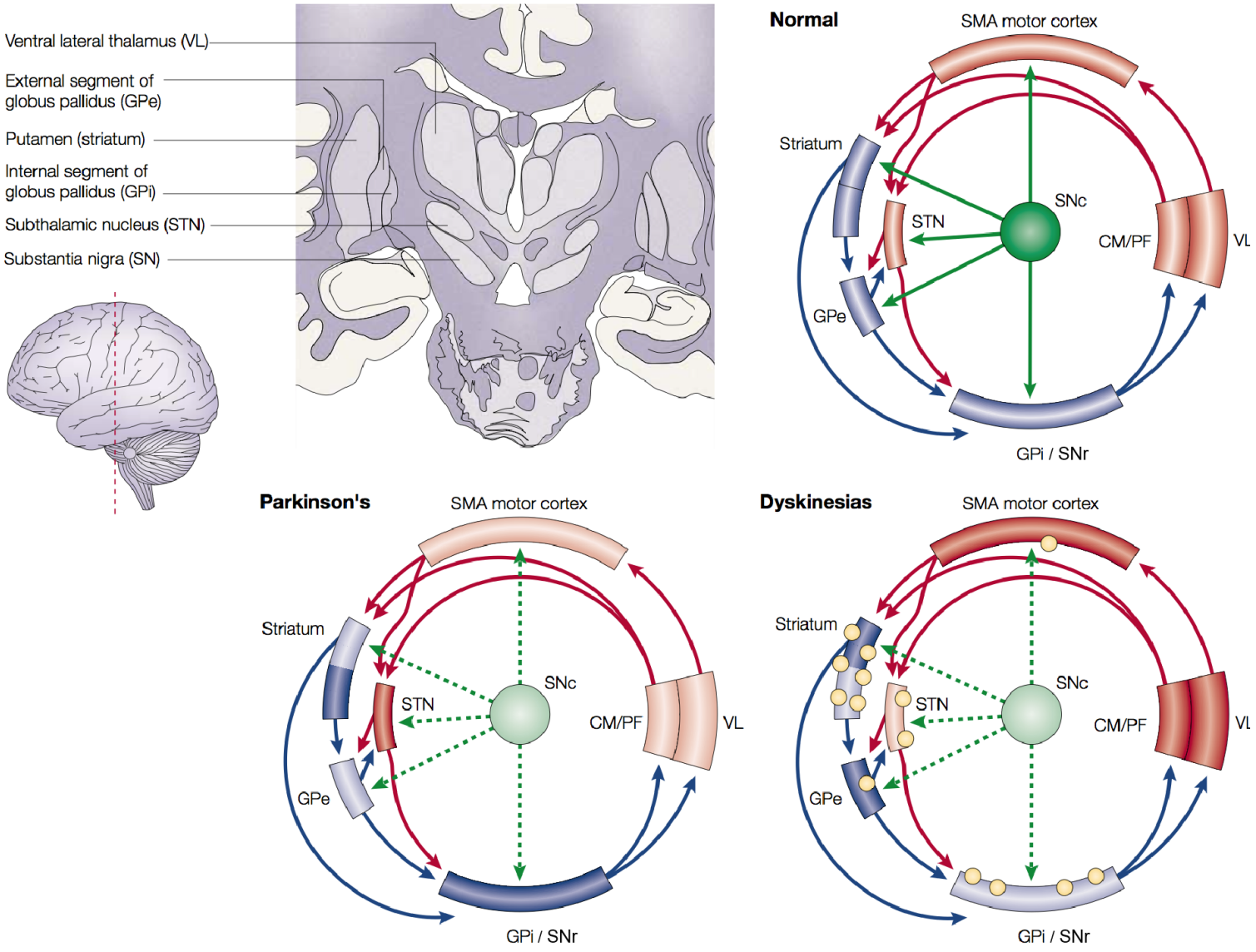

Figure 1. Anatamo-functional connectivity within the basal ganglia–thalamo–cortical circuit in Parkinson's disease and levodopa-induced dyskinesia, from Bezard et al., 2001b. Red corresponds to excitatory glutamate pathways, blue corresponds to inhibitory GABA (γ-aminobutyric acid)-releasing pathway, and green corresponds to the dopamine projections from the substantia nigra pars compacta (SNc). Changes in colour intensity indicate the level of activity of individual projection systems. The upper half of the striatum that is directly connected to the GPi/SNr corresponds to the medium spiny neurons that bear D1 dopamine receptors (direct pathway). The lower half, which is indirectly connected to GPi/SNr through GPe and STN, corresponds to medium spiny neurons that bear D2 dopamine receptors (indirect pathway). In Parkinson's disease, degeneration of SNc neurons (dashed lines) breaks the striatal dopamine homeostasis, inducing hyperactivity of the GPi (darker blue) which brakes neuronal activity in the supplementary motor area (paler red). In levodopa-induced dyskinesia, exogenous supply of levodopa and/or dopamine receptor agonists might act at structures previously innervated by dopamine neurons. So, it is possible that these hypersensitive dopamine receptors (yellow dots) participate in the generation of dyskinesia. The net result would be hypoactivity of the GPi (paler blue), leading to a hyperactivity of SMA neurons (darker red). Deep brain stimulation (DBS) of the GPi or STN has become an efficient method to treat PD patients with severe motor fluctuations and LID. SMA, supplementary motor area; CM/PF, centromedian and parafascicular thalamic nuclei.

3. Spectrum of LID

3.1. Clinical presentation

Eighty to ninety percent of PD patients suffer from LID after ten years of DA replacement therapy (Ahlskog and Muenter, 2001; Hauser *et al.*, 2007). LID can be classified into peak dose dyskinesia (involuntary movements that coincide with the period of best mobility), diphasic dyskinesias (involuntary movements that emerge just before the DA replacement therapy turns the patient "ON" and that reappear at the end of the therapeutic benefit) and "OFF" period dystonia.
Most common movement disorders associated with LID are chorea, dystonia and ballism.

Chorea is characterized by involuntary, irregular, purposeless, nonrhythmic, abrupt and rapid movements that seem to flow from one part of body to the other. Choreic or choreoathetotic movements are the most common forms of LID. They are most commonly associated with peak dose dyskinesia. Chorea usually manifests first on the side of the body that is predominantly affected by PD. The severity of choreic movements varies from minor (involontary movements may not be recognized by patients) to major (involuntary movements may significantly interfere with activities of daily living).

Dystonia is the second most common form of LID. It is characterized by sustained contractions of agonist and antagonist muscles that may involve focal/segmental muscle groups or being generalized. Dystonia as part of LID can be observed as peak dose dyskinesia, diphasic dystonia or "OFF" dystonia. Diphasic dystonia usually presents as painful contractions of the lower limbs that appear for several minutes just before the DA replacement therapy turns the patient "ON" and reemerge at the end of the therapeutic benefit. "OFF" period dystonia symptoms is most commonly seen as painful early morning dystonia of one foot or toes.

Ballism is characterized by very large amplitude unilateral or bilateral choreic movements of the proximal parts of the limbs. Ballistic movements are usually part of severe choreoathetosis rather than being isolated.

3.2. DA replacement therapy in PD

More than 50 years after its introduction (Cotzias *et al.*, 1967), L-Dopa remains the most effective and best tolerated treatment for PD motor symptoms (Fox *et al.*, 2011; Goetz *et al.*, 2005). DA agonists have also proven their efficacy PD in large randomized trials as monotherapy in early PD and adjunt therapy in advanced disease stages (Fox *et al.*, 2011; Goetz *et al.*, 2005). The main interest in prescribing DA agonists in young PD patients (< 65 to 70 years) relies in the retardation of the occurrence of motor complications, such as wearing off and dyskinesia (Hubble, 2002).

Other treatments exist for PD such as inhibitors of the DA degrading enzymes catechol-O-methyl transferase (COMT) and monoamine oxydase (MAO). The efficacy of the COMT inhibitors tolcapone and entacapone have been extensively studied in PD patients with motor fluctuations (Adler *et al.*, 1998; Myllyla *et al.*, 2001; Olanow *et al.*, 2004; Poewe *et al.*, 2002; Rajput *et al.*, 1997; Shoulson *et al.*, 2002; Waters *et al.*, 1997). In clinical trials, new or worsening of dyskinesia was more frequently reported as side effect in patients receiving COMT inhibitors. However, dyskinesia generally subsided after levodopa dose reduction and no differences were observed at study end between COMT inhibitors and placebo in most studies. Rasagiline, an inhibitor of MAO-B, is also used as monotherapy in early PD and add-on in more advanced disease stages (Parkinson Study Group, 2005).

3.3. Genetics of LID

The booming genetics of PD has also rejuvenated the search for forms more susceptible in developing LID. For instance, PARK2 (*parkin*), PARK6 (*pink-1*) and PARK7 (*DJ-1*) are associated with young-onset PD and frequent appearance of dyskinesia suggesting the involvement of genetic factors related to parkinsonism (Dekker *et al.*, 2003). These forms of genetic parkinsonism tend to affect individuals at a younger age, known to be a risk factor for developing LID. However, recent observations suggest that *parkin*–related parkinsonism is delaying the onset of dyskinesia, probably due to a lower daily levodopa dose (Lohmann *et al.*, 2009). Patients with *LRRK2* gene mutations (PARK8) had a higher rate of dyskinesia compared to genetically undefined patients in two studies (Lesage *et al.*, 2008; Nishioka *et al.*, 2010), while this difference was not observed in Israeli LRRK2 patients with

parkinsonism and the cohort of the International LRRK2 Consortium (Healy *et al.*, 2008; Yahalom *et al.*, 2012). Noteworthy, the time to LID onset was longer in patients with mutations in LRRK2 than in patients with idiopathic PD in the cohort of the International LRRK2 Consortium but not in Israeli *LRRK2* patients. It remains therefore unclear if these genetic abnormalities have a direct effect on the risk of developing LID or if other mechanisms play a role.

Besides these genetic forms of PD with a peculiar feature vis-à-vis LID, more classic loci were investigated over the years. Various studies have investigated genetic associations of DA and non-DA receptors implicated in basal ganglia function with LID, as well as DA transporters and enzymes involved in the metabolism of DA. Accordingly, polymorphisms of DA D2 but not of D1 receptors seem to reduce the risk of developing LID (Oliveri *et al.*, 1999; Rieck *et al.*, 2012). By contrast, the TaqIA polymorphism located in the gene encoding the D2 receptor was shown to increase the risk of developing motor fluctuations in PD patients (Wang *et al.*, 2001). This observation was not confirmed by others (Lee *et al.*, 2011; Rieck *et al.*, 2012). In a study investigating genetic susceptibility factors of diphasic and peak-dose LID, diphasic dyskinesia was associated with the DA D3 receptor p.S9G variant. Carrying the AA genotype was likely to shorten the onset of diphasic dyskinesia, while the presence of peak dose LID was not associated with any of the genetic variants studied (Lee *et al.*, 2011). One recent study found that a polymorphism in the *SLC6A3* gene encoding for the DA transporter extends the time to LID onset (Kaplan *et al.*, 2014). Finally, a COMT Val158Met polymorphism is associated to an increased risk of developing dyskinesia (de Lau *et al.*, 2012).

Opioid receptors have also been implicated in the pathophysiology of LID. Strong et al. found that carrying the G-allele of the A118G single nucleotide coding region polymorphism of the mu opioid receptor is associated with an increased risk of earlier onset of dyskinesia (Strong *et al.*, 2006).

The role of brain derived neurotrophic factor (BDNF) on LID has also been investigated. PD patients with the met allele of BDNF have a significantly higher risk of developing dyskinesia earlier in the course of their disease (Foltynie *et al.*, 2009). However, a more recent study did not observe an association between seven polymorphisms of the *BDNF* gene and LID onset (Kaplan *et al.*, 2014).

Taken together, the contribution of the genetic factors in the overall risk of developing LID needs further investigation or could be considered as of modest impact upon the propensity of patients to develop LID. New findings may however have implications for helping predict individual PD patients at risk of developing LID.

3.4. Current management of LID in PD patients

3.4.1. Current treatments

3.4.1.1. Amantadine

Amantadine is a NMDA antagonist known for many years to have a modest antiparkinsonian action (Fox *et al.*, 2011). The efficacy of amantadine at reducing dyskinesia severity was first assessed during an acute intravenous levodopa infusion in a small placebo-controlled cross-over study in 18 PD patients with motor fluctuations and peak-dose dyskinesia (Verhagen Metman *et al.*, 1998c). Amantadine reduced peak-dose dyskinesia severity without modifying PD motor symptoms. The level of dyskinesia reduction was related to plasma amantadine concentration. The same patients were assessed one year later in a placebo-controlled follow-up paradigm while still receiving amantadine (Metman *et al.*, 1999). The magnitude of the antidyskinetic effect was similar suggesting a sustained effect of amantadine on peak-dose dyskinesias. Another randomized placebo-controlled study tested the effectiveness of amantadine in 18 consecutive PD patients with motor fluctuations and peak-dose dyskinesia (da Silva-Junior *et al.*, 2005). After three weeks of treatment, Unified Parkinson's Disease Rating Scale (UPDRS) scores were improved while Clinical Dyskinesia Rating Scale (CDRS) scores were unchanged and not different from placebo. The effect of acute intravenous amantadine infusion was also tested in 9 PD patients with motor fluctuations and peak-dose dyskinesia in a placebo-controlled cross-over study (Del Dotto *et al.*, 2001). Intravenous amantadine infusion reduced AIMs scores by 50% compared to placebo.

The observation of a benefit of amantadine treatment lasting for less than 8 months in a randomized, placebo-controlled study including 40 PD patients with motor fluctuations and peak-dose dyskinesia questioned the usefulness of this drug for treating dyskinesia in the long run (Ory-Magne *et al.*, 2014; Thomas *et al.*, 2004). Withdrawal of the drug at study end induced a transient rebound with increase of dyskinesia in 11 patients.

The results of the trial by Thomas et al. (2004) was recently challenged (Thomas *et al.*, 2004) by two randomized placebo-controlled parallel-group studies that assessed the long-term antidyskinetic effect of amantadine in 32 and 57 PD patients, respectively (Ory-Magne *et al.*, 2014; Wolf *et al.*, 2010). In the first study, patients who received amantadine for LID for at least one year were switched in a double blind manner to amantadine or placebo. Dyskinesia scores increased significantly in patients receiving placebo but not in those pursuing amantadine. In the second trial, patients were switched to either amantadine or placebo after at least six months of stable treatment with amantadine (Ory-Magne *et al.*, 2014). Similar to the study by Wolf and colleagues (Wolf *et al.*, 2010), dyskinesia scores were significantly higher in the placebo group compared to patients pursuing amantadine. Moreover, dropouts for LID worsening and higher AIMs scorer were observed in the discontinuing group.
Taken together, the results of both recent trials argue for long-term antidyskinetic effects of amantadine in PD patients with LID.

3.4.1.2. Deep brain stimulation (DBS)

Within the last two decades, DBS of the Subthalamic Nucleus (STN) and the internal part of the Globus Pallidus (GPi) (**Figure 1**) have become routine methods for treating PD patients with severe motor fluctuations and LID (Deuschl *et al.*, 2006; Follett *et al.*, 2010; Krack *et al.*, 2003; Moro *et al.*, 2010; Volkmann, 2004; Weaver *et al.*, 2012).

DBS of the postero-ventral part of the GPi is particularly effective to treat LID. Accordingly, a reduction of 76% of LID severity until 35 months after surgery was observed (Rodrigues *et al.*, 2007). A longer follow-up confirmed marked and sustained improvement of dyskinesia (75% in duration and 64-100% in severity) over 5-6 years of pallidal stimulation (Moro *et al.*, 2010; Volkmann, 2004). The efficacy of pallidal stimulation is independent of the type of LID and also includes respiratory dyskinesias (Oyama *et al.*, 2011). Although the underlying mechanisms remain unclear some evidence suggests that the reduction of LID could be related to the stimulation of inhibitory afferents coming from the striatum, the external segment of the Globus Pallidus (GPe) or collaterals of GPi neurons to the posterior ventral pallidum (Boraud *et al.*, 1996; Wu *et al.*, 2001). Alternatively, GPi-DBS could reverse the abnormal pattern of neuronal activity that is induced by DA replacement treatment in the basal ganglia cortex network (**Figure 1**) (Guridi *et al.*, 2008; Wu *et al.*, 2001).

STN-DBS has positive effects on LID, but may also induce involuntary choreic or ballistic movements. The occurrence of choreic or ballistic movements in the operating room is even considered as an indication for the accurate placement of the stimulation leads within the sensorimotor part of the STN. STN-DBS induced involuntary movements are frequent during the first month following surgery (Zheng *et al.*, 2010). Sometimes, they persist and make additional GPi-DBS necessary (Reese *et al.*, 2011). In most cases, the duration of LID decreases over time (Deuschl *et al.*, 2006; Follett *et al.*, 2010; Krack *et al.*, 2003; Simonin *et al.*, 2009). The shift from 130 Hz to 80 Hz may be helpful in patients with residual LID (Merola *et al.*, 2013). STN-DBS improves the entire spectrum of LID including peak dose, biphasic and off period dyskinesias (Fraix *et al.*, 2010; Katayama *et al.*, 2006; Krack *et al.*, 1999). The mechanisms involved in the reduction of LID after STN-DBS remain unclear but the concomitant decrease in DA replacement therapy is frequently incriminated (Follett, 2004; Kim *et al.*, 2008; Krack *et al.*, 1997; Russmann *et al.*, 2004). Indeed, STN-DBS reduces LID by 46–85%, which is paralleled by a concomitant reduction of the equivalent daily levodopa dose by 50% (Breit *et al.*, 2004; Guridi *et al.*, 2008). The delayed decrease in LID further suggests that the desensitization to LID requires several months of drug withdrawal (Russmann *et al.*, 2004). However, some authors suggested a direct effect of DBS on STN neurons and the structures in the vicinity of this nucleus. Accordingly, DBS dorsal to the STN (zona incerta, lenticulus fascicularis) seems to be able to directly suppress LID independently of changes in DAergic medication (Alterman *et al.*, 2004; Herzog *et al.*, 2003). DBS of this region may improve LID through a disruption of the pallido-thalamic connection or a modification of the activity pattern of STN neurons (Garcia *et al.*, 2003; Katayama *et al.*, 2006; Meissner *et al.*, 2005; Obeso *et al.*, 2000; Sankar and Lozano, 2011). Others have hypothesized that STN-DBS induces overall stabilization of the basal ganglia network and striatal synaptic function (**Figure 1**) (Simonin *et al.*, 2009).

Taken together, GPi- and STN-DBS are effective treatments of LID for PD patients suffering from motor fluctuations and severe form of PD. To date, there is no study that has demonstrated a significant difference in efficacy against either LID or motor symptoms between pallidal and STN-DBS (Follett *et al.*, 2010; Lukins *et al.*, 2014; Odekerken *et al.*, 2013; Sako *et al.*, 2014). Thus, the choice of the target must be determined in a patient-by-patient fashion.

DBS of the thalamic centromedian and parafascicular complex (CM/PF) (**Figure 1**) seems also effective in reducing LID severity in PD (Caparros-Lefebvre *et al.*, 1999).

3.4.2. Strategies under investigation in clinical trials

3.4.2.1. NMDA antagonists

Beyond amantadine, other NMDA antagonists such as dextromethorphan, remacemide, milacemide, CP-101,606 and memantine were assessed for treating LID (Clarke *et al.*, 2001; Giuffra *et al.*, 1993; Merello *et al.*, 1999b; Nutt *et al.*, 2008; Parkinson Study Group, 2001; Shoulson *et al.*, 2001; Verhagen Metman *et al.*, 1998a).

Dextromethorphan was assessed in 18 PD patients with motor fluctuations and LID in a placebo-controlled cross-over trial (Verhagen Metman *et al.*, 1998a). 12 patients were excluded because of decreased levodopa efficacy or no benefit at the highest dose. In the remaining six patients who were subjected to the placebo-controlled cross-over phase, dextromethorphan decreased the severity and duration of dyskinesia and severity of motor fluctuations.

Memantine was tested in 12 PD patients with motor fluctuations and LID in a placebo-controlled cross-over study (Merello *et al.*, 1999b). UPDRS "ON" and "OFF" motor scores were decreased in patients receiving memantine, while dyskinesia ratings were unchanged.

Remacemide efficacy, a non-competitive NMDA channel antagonist, has been evaluated by the Parkinson Study Group in two randomized, placebo-controlled, parallel group study trials. In a pilot study, doses ranging between 150 and 600 mg of remacemide were tested against placebo in 39 PD patients with motor fluctuations and disabling LID (Parkinson Study Group, 2001). There were no differences between the placebo and remacemide group for any of the dyskinesia measures. Adverse events mainly occurred in the group receiving 600mg remacemide. In a second large scale trial, remacemide was tested against placebo in 279 PD patients with motor fluctuations who experienced more than 25% of the waking day in the "OFF" state (Shoulson *et al.*, 2001). UPDRS motor scores were improved in patients receiving remacemide (only 150 and 300 mg) compared with placebo. No dyskinesia ratings were performed.

CP-101,606 is a selective antagonist of the GluN2B subunit of the NMDA receptor that was assessed in 12 PD patients with motor fluctuations and dyskinesia in a randomized, placebo-controlled cross-over study (Nutt *et al.*, 2008). Patients received either CP-101,606 (low or high dose) or placebo during intravenous levodopa infusion. Both doses of CP-101,606 similarly reduced dyskinesia scores compared to placebo, while UPDRS motor scores were not different between groups. Many patients receiving CP-101,606 presented dose-dependently abnormal thinking, depersonalization and amnesia.

Budipine is an NMDA antagonist with widespread action on other neurotransmitter systems. This drug was tested in 7 PD patients with motor fluctuations in an open-labeled trial (Spieker *et al.*, 1999). Motor scores improved and "OFF" time decreased without appearance of dyskinesia in most patients. Larger randomized, controlled clinical studies were stopped or planned trials were not conducted when a prolongation of the QT interval in the ECG was observed with the risk of fatal polymorphic ventricular tachycardia.

Milacemide, a glycine prodrug that positively modulates NMDA transmission, was tested in a placebo-controlled cross-over study in 6 PD patients with motor fluctuations (Giuffra *et al.*, 1993). Milacemide worsened parkinsonian motor signs, mainly rigidity, without any effect on dyskinesia ratings.

3.4.2.2. mGLUR5 negative allosteric modulators

The mGluR5 antagonists and negative allosteric modulators (NAMs) have emerged as a novel and potentially highly desirable class of compounds for the treatment of LID. Few trials have been conducted so far. In two clinical phase II trials investigating safety and efficacy of the mGluR5 marvoglurant, AFQ056, demonstrated a significant reduction of LID in PD patients in only one (Stocchi *et al.*, 2013) out of two studies (Kumar *et al.*, 2013). Kumar et al. however found a reduction of OFF time (Kumar *et al.*, 2013). Phase III data have yet to be published but were communicated by the company as negative.

Dipraglurant, another potent mGluR5 NAM (Duvey *et al.*, 2013), was tested in a Phase 2A proof-of-concept 4-week, randomized, double-blind, placebo-controlled, parallel-group clinical trial in PD patients with moderate or severe LID, and proved to significantly reduce

severity of LID, although full disclosure of data in a peer-reviewed publication is still awaited. Larger trial or repetition of this small pilot study is awaited.

3.4.2.3. Antiepileptics

The efficacy of gabapentine on motor severity and activities of daily life was first tested in 19 PD patients with motor fluctuations and dyskinesia in a randomized placebo-controlled cross-over study (Olson *et al.*, 1997). Total UPDRS scores were lower in patients receiving gabapentine,

The effect of gabapentine (up to 2400mg/d) on motor complications was more specifically investigated in a second randomized, placebo-controlled cross-over trial including 15 PD patients with motor complications and dyskinesia (Van Blercom *et al.*, 2004). Secondary outcome measures included dyskinesia measures. No differences were observed between gabapentine and placebo. Dizziness and accidental falls were more frequent in patients receiving gabapentine.

The antidyskinetic properties of levetiracetam were assessed in two small open-label studies (Lyons and Pahwa, 2006; Zesiewicz *et al.*, 2005). One study reported an increase in "ON" time without or with non-troublesome dyskinesia by 18% in 9 PD patients with peak-dose dyskinesia for at least 25% of waking hours who received up to 3,000mg/d of levetiracetam (Zesiewicz *et al.*, 2005). At the same time, "ON" time with troublesome dyskinesia decreased by 12%. There was a considerable dropout rate with a withdrawal of 56% of the patients, mostly because of somnolence. The second study was also conducted in 9 PD patients experiencing moderate to severe dyskinesia and receiving up to 3,000mg/d of levetiracetam (Lyons and Pahwa, 2006). This study reported a dropout of 44%, mostly due to worsening of PD symptoms or somnolence. Moreover, of the remaining 5 patients, 4 discontinued levetiracetam after the end of the study because of worsening of PD symptoms and somnolence. Several larger trials were performed or are ongoing (cf. http://clinicaltrials.gov). Theirs results still await publication.

Zonisamide (25-100mg) was tested in a randomized, placebo-controlled, parallel-treatment study including 347 PD patients with motor fluctuations (Murata *et al.*, 2007). Zonisamide

(50mg) decreased disabling dyskinesia. However, patients receiving zonisamide complained dose-dependently about more dizziness, apathy and a decrease in body weight.

3.4.2.4. Antipsychotics

The efficacy of clozapine, a DA receptor antagonist with anti-serotoninergic, anti-muscarinic, anti-adrenergic and anti-histaminergic properties, in decreasing LID was evaluated in several small pilot studies (Bennett *et al.*, 1994; Bennett *et al.*, 1993; Durif *et al.*, 1997; Pierelli *et al.*, 1998) and in one larger randomized, placebo controlled trial (Durif *et al.*, 2004). In the latter, patients under clozapine gained 2.4h of "ON" time without dyskinesia compared to placebo. There was no increase in the duration of "OFF" periods. Dyskinesia ratings at rest were decreased during the acute levodopa challenge. However, ratings in the same condition during an activation task were not different. Clozapine had no effect on the antiparkinsonian action of levodopa. Adverse events were not more frequent with clozapine except for drowsiness and hypereosinophilia, the latter rapidly resolved after treatment discontinuation.

Olanzapine has shown antidyskinetic properties in a small randomized, placebo-controlled cross-over trial (Manson *et al.*, 2000b). However, adverse events were more common with olanzapine, consisting in increased "OFF" time, increased parkinsonism and increased drowsiness.

Quetiapine, another atypical antipsychotic with few extrapyramidal side effects, was tested in a small randomized, placebo-controlled, cross-over study enrolling 8 PD patients with disabling LID (Katzenschlager *et al.*, 2004). No differences were observed between quetiapine or placebo. The double blind trial was followed by an open-label period of around 30 days during which patients received up to 50mg/d of quetiapine. Mild improvement in dyskinesia duration and severity were observed during the open-label period according to patient home diaries.

3.4.2.5. Serotonin 5-HT_{1A} agonists

Sarizotan was assessed in 18 PD patients with motor fluctuations and peak-dose LID in a randomized pilot study (Bara-Jimenez *et al.*, 2005). Sarizotan (5 mg) decreased LID by 40% and increased levodopa half-life time values by 38%. The drug failed to improve UPDRS motor scores.

In a randomized, placebo-controlled, dose finding trial, 398 PD patients with at least moderately disabling LID for at least 25% of the waking day either received sarizotan 2, 4, 10 mg/d or placebo (Goetz *et al.*, 2007). Mean improvements in "ON" time without dyskinesia were not different between groups. The analysis of the patient home diaries did not reveal any differences in "ON" time measures (with and without dyskinesia, with and without non-troublesome dyskinesia). Modified AIMs scores at rest and with activity were not different between groups, while patients receiving 2mg/d sarizotan had lower UPDRS scores. No adverse events were more common in patients treated with sarizotan compared to placebo except for an increase in "OFF" time in patients receiving 10mg/d sarizotan and a similar trend for 4mg/d.

Oral administration of the serotonin receptor type 1A agonist buspirone prior to levodopa reduced levodopa-evoked striatal synaptic dopamine increases and attenuated LID in a recent small scale investigational study (Politis, 2014), somewhat confirming earlier results in other small scale trials (Bonifati *et al.*, 1994; Kleedorfer *et al.*, 1991).

3.4.2.6. Other strategies

Cannabis was examined in a randomized, placebo-controlled cross-over design in 19 PD patients with LID (Carroll *et al.*, 2004). Cannabis tended to worsen dyskinesia. No serious adverse events were observed.

Nabilone, a cannabinoid, has shown antidyskinetic properties in a small randomized, placebo-controlled cross-overt study in 7 PD patients who experienced LID during 25-50% of waking hours (Sieradzan *et al.*, 2001). Nabilone decreased dyskinesia by 22.2% compared to placebo, while the duration of "ON" time and the percentage of dyskinesia during "ON" time was not modified. Nabilone had no effect on the antiparkinsonian action of levodopa. Two patients were withdrawn from the study because of side effects (vertigo, orthostatic hypotension).

The opioid antagonist naltrexone was assessed in 10 PD patients with end-of-dose wearing "OFF" and 8 PD patients with dyskinesia in a randomized trial (Rascol *et al.*, 1994). Naltrexone had no effect on motor function or dyskinesia severity and duration. Adverse events (digestive, neuropsychiatric) were more frequent in patients under naltrexone.

The anti-dyskinetic properties of the α2-adrenoreceptor antagonist idazoxan were evaluated in a single oral dose randomized, placebo-controlled study in 18 PD patients with peak-dose dyskinesia (Rascol *et al.*, 2001). There was a trend for lower dyskinesia in patients receiving 10 or 20mg idazoxan. Cardiovascular adverse events were more frequent with idazoxan. A randomized cross-over trial in 7 PD patients failed to show an effect of idazoxan on LID (Manson *et al.*, 2000a). No differences were observed between idazoxan and placebo in terms of motor function and dyskinesia severity. All patients experienced side effects during idazoxan treatment which were serious enough in 3 to discontinue study medication.

A ten day treatment with transdermal high dose 17[beta]-estradiol (0.4 mg/d) was studied in 8 female PD patients with LID in a randomized, placebo-controlled cross-over study (Blanchet *et al.*, 1999). The threshold dose of levodopa to provide antiparkinsonian efficacy was reduced. By contrast, the duration of the clinical motor response and dyskinesia ratings were not different between groups. While on estradiol, most patients complained of breast/nipple tenderness and three patients reported increased dyskinesia.

3.5. Beyond LID: impulse control disorders and DA dysregulation syndrome

In addition to the wide array of non-motor symptoms occurring in PD, DA replacement therapy (DRT) can induce non-motor side-effects. Among them, addiction-like disorders have been described with a growing interest since the early 2000's (Giovannoni *et al.*, 2000; Lawrence *et al.*, 2003; Weintraub and Potenza, 2006). They mainly encompass impulse control disorders (ICD), which can be seen as behavioral addictions and DA dysregulation syndrome (DDS), corresponding to compulsive medication use. While they mainly have a non-motor expression, their link with LID has been questioned (Voon *et al.*, 2009).

3.5.1. Impulse control disorders (ICD)

ICDs are behavioral affections during which individuals fail to resist to internal or external stimuli, leading them to act inconsiderately. They generate anxiety and can result in dramatic alterations of the social or professional functioning (Pontone *et al.*, 2006). The clinical spectrum of ICD has been extensively described elsewhere (Voon *et al.*, 2009; Voon *et al.*, 2007a). Briefly, different types of ICD are reported.

3.5.1.1. Pathological gambling

Pathological gambling is the most common ICD and is found in 5 to 6% of PD patients (Avanzi *et al.*, 2006; Weintraub *et al.*, 2010). Parkinsonian gamblers are mainly interested in games providing immediate reward such as scratch cards, bets, casinos or internet gambling games (Voon *et al.*, 2009). Pathological gambling can have dramatic consequences on patient's life due to important monetary losses, with an average financial loss of US$10000 (Voon *et al.*, 2006).

3.5.1.2. Compulsive shopping

Compulsive shopping is defined by continuous thoughts toward buying behavior and results in high anxiety (Black, 2007). In PD, compulsive shopping is observed in 5.7% of patients (Weintraub *et al.*, 2010) but such troubles may be difficult to detect and necessitate the use of defined diagnostic criteria (McElroy *et al.*, 1994). The purchases are usually useless, very expensive, time-consuming and anxiogenic and should be observed aside from any maniac episode. Compulsive shopping seems to be more commonly observed in women than in men (7.8% vs. 4.5% (Weintraub *et al.*, 2010)).

3.5.1.3. Hypersexuality

Hypersexuality has been defined by the presence of maladaptive preoccupation with sexual thoughts with the occurrence of intrusive paraphiliac ideations preventing the focusing on daily tasks. Orgasms are described as not satisfying and lead to the need of repeated sexual intercourses (Kaplan, 1994). Its prevalence is difficult to estimate but might reach 3.5% (Weintraub *et al.*, 2010) and diagnostic criteria have been proposed (Voon *et al.*, 2006). Hypersexuality appears to occur more frequently in men than in women (5.2% vs. 0.5% (Weintraub *et al.*, 2010)). Compulsive sexual behaviors are difficultly tolerated by PD patients and are often associated with depression (Klos *et al.*, 2005; Voon *et al.*, 2006). Moreover, these behaviors can have disastrous consequences on the spouse or a third person (Muller *et al.*, 2013).

3.5.1.4. Compulsive eating

Compulsive eating refers to an irresistible need of food intake far beyond satiety (Nirenberg and Waters, 2006). Its occurrence in PD is estimated to 4.3% (Weintraub *et al.*, 2010). Unlike the majority of parkinsonian patients who are losing weight, an abnormal weight increase is usually a red flag (Nirenberg and Waters, 2006). Diagnostic criteria follow the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) items and include: the occurrence of binge eating along with a loss of control, rapid eating, feeling uncomfortably full, eating large amounts when not hungry, eating alone because of embarrassment of amounts, feeling disgusted or guilty after overeating, in the presence of visible distress. Such behaviors should be present during at least 2 days/week over 6 months in absence of compensatory behaviors, or during anorexia or bulimia nervosa (American-Psychiatric-Association, 2000).

3.5.1.5. Punding

Punding is not considered as an ICD *per se* but frequently follows the same classification because of its compulsive nature. Initially described in amphetamine and cocaine users (Rylander, 1972; Schiorring, 1981), punding can be defined as an intense fascination for repetitive tasks, which can be simple such as gathering, manipulating, sorting objects or more complex such as painting or gardening (Voon *et al.*, 2009). Punding is often misevaluated in PD because of the lack of precise diagnostic criteria, but studies reported a prevalence between 1.4 and 14% (Evans *et al.*, 2004; Miyasaki *et al.*, 2007). Punding is mainly observed with apomorphine and D2/3 agonists.

Finally, the spectrum of DRT-induced ICD can extend to various other behaviors such as excessive hoarding, kleptomania or reckless generosity (Bonfanti and Gatto, 2010; O'Sullivan *et al.*, 2010a; O'Sullivan *et al.*, 2010b).

Clinical studies reported the occurrence of ICD within 24 months following the beginning of the treatment and a cessation of the troubles when DRT is tapered or stopped (Dodd *et al.*, 2005). They are preferentially observed with DA D2/3 receptor agonists (Gallagher *et al.*, 2007), which might increase by 2 to 3.3 fold the risk of developing such troubles (Weintraub *et al.*, 2010). However, this latter study highlighted than an adjunctive L-dopa therapy increases the odds of an ICD by 50% compared to DA agonists alone. A cross-sectional study conducted in the United States and Canada identified the occurrence of at least one ICD in

13.6% of PD patients (Weintraub *et al.*, 2010). A younger age at disease onset, past experiences of illegal drug use or cigarette smoking might constitute risk factors (Bastiaens *et al.*, 2013; Weintraub *et al.*, 2010). A novelty seeking (Voon *et al.*, 2007b) or a sensation seeking (Djamshidian *et al.*, 2011a) personality have also been reported and are suspected to be important factors in the development of ICD.

3.5.2. DA dysregulation syndrome (DDS)

DDS refers to a pathological overconsumption of the DAergic medication. Its occurrence is estimated between 3 and 4% of PD patients (Giovannoni *et al.*, 2000; Pezzella *et al.*, 2005), is mainly found in patient with younger age at disease onset and is associated with past legal or illegal drug use (Evans *et al.*, 2005; Giovannoni *et al.*, 2000; Lawrence *et al.*, 2003). In addition, most DDS cases are reported in patients taking fast-acting drugs such as subcutaneous apomorphine or L-Dopa as opposed to long acting drugs (Giovannoni *et al.*, 2000). Despite an appropriate treatment, DDS patients feel under-medicated and start to increase their DRT intake (Lawrence *et al.*, 2003). This is associated with a distorted perception of the motor status, and patients only feel 'on' when highly dyskinetic (Giovannoni *et al.*, 2000; Lawrence *et al.*, 2003). Moreover, a sensation of pleasure, well-being and a psychostimulant effect are reported (Castrioto *et al.*, 2013; Tellez *et al.*, 2006). A core feature of DDS is an increased intake of DRT, which will necessitate multiple providers (multiple physicians, internet purchases) or drug hoarding (Lawrence *et al.*, 2003; Tellez *et al.*, 2006). Thus, DDS has been associated to a 'hedonistic homeostatic dysregulation' (Giovannoni *et al.*, 2000) where the motivation to retrieve the pleasant feelings procured by the drug is driven by the unpleasant sensation of withdrawal (Koob and Le Moal, 1997; Solomon and Corbit, 1973). DDS is associated with the presence of LID and ICD (Giovannoni *et al.*, 2000).

The DDS diagnosis follows the DSM-IV criteria for substance dependence (American-Psychiatric-Association, 2000) but its use has been discussed in the specific context of PD (Bearn *et al.*, 2004). Indeed, the excessive DRT intake is present in the context of a neurologic disease, which needs drug intake to relieve motor symptoms (Giovannoni *et al.*, 2000). In this regard, an alternative classification has been proposed. Mainly following the DSM-IV criteria, it considers the specificities of PD and propose the diagnostic of a DDS when symptoms vary from a 'classical' parkinsonian syndrome (Giovannoni *et al.*, 2000).

3.5.3. DA replacement therapy withdrawal syndrome

In patients presenting DDS, tapering or stopping L-Dopa or apomorphine resulted in withdrawal signs including dysphoria, depression, irritability and anxiety (Giovannoni *et al.*, 2000; Lawrence *et al.*, 2003). These negative sensations are different from those classically occurring for end-of-dose wearing-off signs and have been considered as core features of DDS diagnostic. More recently, withdrawal signs have also been observed in up to 19% of PD patients specifically treated with DA agonists (Rabinak and Nirenberg, 2010). They are similarly characterized by psychostimulant-like withdrawal symptoms such as anxiety, irritability, orthostatic hypotension and panic attack when the treatment is reduced and have been named DA agonist withdrawal syndrome (DAWS). Wearing-off symptoms refractory to L-Dopa and psychiatric manifestation resistant to antidepressant or anxiolytic are specific features of DAWS. Interestingly, DAWS has exclusively been observed in patients presenting ICD (Pondal *et al.*, 2013; Rabinak and Nirenberg, 2010), suggesting a generalized reward dysfunction.

3.5.4. Dyskinesia and compulsive behaviors

The repetitive movements of dyskinesia and the compulsive behaviors of punding have been proposed to share common mechanisms (Voon *et al.*, 2009). An altered functioning of the basal ganglia network is associated with deficits in inhibiting competing behaviors (Mink, 1996). Clinical studies showed that PD patients with severe motor symptoms have deficits in on-line suppression of impulsive responses (Wylie *et al.*, 2010). Moreover, parkinsonian patients presenting punding behaviors exhibit more severe dyskinesia than other patients and punding severity correlates with dyskinesia severity (Silveira-Moriyama *et al.*, 2006). Finally, LID and compulsive DRT use might share similar presynaptic mechanisms. Studies using [^{11}C]raclopride (a D2/D3 receptor ligand) binding revealed an increased DA release in the dorsal striatum after L-Dopa intake in dyskinetic patients (de la Fuente-Fernandez *et al.*, 2004b) whereas an increased DA release in the ventral striatum after L-Dopa intake is observed in DDS patients (Evans *et al.*, 2006). Altogether, it appears that both LID and compulsive behaviors are two aspects resulting from abnormal DAergic stimulation. A better understanding of their common mechanisms will be crucial for their treatment.

3.6. Other L-Dopa-induced side effects

Besides LID and L-Dopa-induced compulsive behaviours, several other side effects have been reported, including autonomic symptoms (nausea, orthostatic hypotension), somnolence, and psychiatric complications (hallucinations, delusions). However, some of these autonomic and psychiatric manifestations are also inherent to the disease and their link with L-Dopa is thus not as straightforward as it is the case with LID and compulsive behaviours triggered by L-Dopa.

L-Dopa (as well as DA agonists) can induce nausea and vomiting due to the stimulation of DA receptors of the area postrema (Duvoisin, 1972). The area postrema being devoided of blood-brain barrier, nausea and vomiting are significantly reduced with peripheral inhibitors of dopa-decarboxylase (Lieberman *et al.*, 1975).

Orthostatic hypotension is a well-identified autonomic symptom of PD related to the involvement of the sympathetic nervous system early in the disease process (Fereshtehnejad and Lokk, 2014). Several lines of evidence indicate that L-Dopa can have cardiovascular side-effects, including worsening of orthostatic hypotension (Senard *et al.*, 1997) blood pressure and heart rate decrease (Bouhaddi *et al.*, 2004).

Altered vigilance, including somnolence (excessive daytime sleepiness) and sudden-onset sleep episodes (sleep attacks) is frequent in PD patients. Several studies have demonstrated an association between L-Dopa equivalent dosage and excessive daytime sleepiness (Brodsky *et al.*, 2003; Ghorayeb *et al.*, 2007; Ondo *et al.*, 2001). Intake of L-Dopa has also been associated with increased risk of sleep attacks (Brodsky *et al.*, 2003) and L-Dopa can also induce drowsiness in healthy subjects (Micallef-Roll *et al.*, 2001).

Hallucinations in PD have been historically described prior to the introduction of L-Dopa (Fenelon *et al.*, 2006), although they are classically considered a side-effect of DRT, which may affect up to 40% of PD patients (Fenelon *et al.*, 2000). Several studies have contributed to highlight a multifactorial origin of hallucinations in PD and have consistently identified several risk factors such as advanced age, duration of the disease and cognitive status (Biglan

et al., 2007; Fenelon *et al.*, 2000; Zhu *et al.*, 2013). Although some studies have found links between L-Dopa dosage and the occurrence hallucinations (Zhu *et al.*, 2013), other studies however reported significant association with ergot DA agonists (Williams and Lees, 2005) and/or failed to report a significant association with L-Dopa intake (Merims *et al.*, 2004; Williams and Lees, 2005). Even though DAergic treatments as a whole can be considered as one of many risk factors for hallucinations in PD, there is no definite evidence incriminating L-Dopa rather than other antiparkinsonian therapies.

4. Animal models of LID

Understanding the pathophysiology of LID for then proposing evidence-based therapeutic solutions has triggered a continuous search for adequate experimental models of the L-Dopa-induced side effects in animals.

4.1. LID in the reserpine-treated rat model of PD

The reserpine model was the first animal model of PD. Carlsson and co-workers in 1957 showed for the first time that the central action of reserpine induces a sharp decrease in motor activity with resultant hypokinesia, akinesia and even catalepsy in several species (Carlsson *et al.*, 1957). Animals also present other symptoms, which resemble those observed in human PD, the most frequent being rigidity of skeletal muscles, tremor and postural flexion. Several studies were conducted on this PD animal model receiving L-Dopa to investigate the potential of various agents to reduce LID. Behaviour was assessed using an automated movement detection system, allowing to estimate horizontal activity, and vertical activity. In the reserpine-treated rat model, administration of a high dose of L-dopa (150 mg/kg) produced a hyperkinetic state characterized by an increase in horizontal and vertical activity, which were proposed to represent correlates of antiparkinsonian and prodyskinetic activity, respectively. Some drugs that have previously been found to reduce LID in parkinsonian primates and PD patients without compromising the anti-parkinsonian efficacy of L-Dopa selectively and dose-dependently reduce vertical components of activity when co-administered with L-Dopa in the reserpine-treated rats (e.g. amantadine and idazoxan). Others, as haloperidol (1 mg/kg), an agent lacking the ability to selectively reduce LID without compromising the antiparkinsonian actions of L-Dopa, reduced both horizontal and vertical activity. Such model, heavily used in

the past, is not anymore included in the mainstream translational chain of models for validating either a putative therapeutic target or a therapeutic strategy for LID.

4.2. Behavioural sensitization in the 6-OHDA-lesioned rat

Sensitization to dopamimetic drugs, i.e. L-Dopa, DA agonists or DA-releasing agents, was first defined as a behavioural phenomenon in a rodent model of PD (Morelli *et al.*, 1989; Ungerstedt, 1971b).

The model consists in rats which ascending DA nigrostriatal neurons are unilaterally destroyed by an intracerebral injection of 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle (MFB). In this model, systemic administration of dopamimetic drugs results in turning (rotation) of the animal towards the side opposite to the lesioned one (contralateral turning) while DA-releasing agents (amphetamine) cause ipsilateral turning (Ungerstedt, 1971b). Such rotational behaviour is thought to result from the supersensitivity of DAergic receptors in the denervated side and requires an extensive denervation for being observable (>95%). It is routinely used for checking beforehand the extent of DA depletion (Schwarting and Huston, 1996a; Schwarting and Huston, 1996b).

4.3. Abnormal involuntary movements in the 6-OHDA-lesioned rodents

4.3.1. Rat Model

For decades, the Ungerstedt model (Ungerstedt, 1971b) has constituted the gold standard of the rodent research in PD until few researchers begun to look at rodents for what they are physically able to perform (for review, see Cenci *et al.*, 2002).

In the late 90's, M.A. Cenci developed the abnormal involuntary movement (AIM) rating in the L-Dopa-treated 6-OHD-lesioned rat (Cenci *et al.*, 1998). She observed that rats were not simply displaying a sensitized rotational behaviour but also a series of complex behaviours that were resembling LID (Cenci *et al.*, 2002). AIMs affect the forelimb contralateral to the lesion (limb dyskinesia), the trunk with twisting movements (axial dyskinesia), the orofacial musculature (orofacial dyskinesia) and a contralateral circling locomotive behaviour (e.g.

Cenci *et al.*, 1998; Lee *et al.*, 2000a; Lundblad *et al.*, 2002; Winkler *et al.*, 2002). These AIMs are quantified on the basis of their topographical distribution, amplitude and duration (e.g. Cenci *et al.*, 1998; Lee *et al.*, 2000a; Lundblad *et al.*, 2002; Winkler *et al.*, 2002), as done in the clinic for rating LID. For each of these AIMs categories, all the rats are scored using a scale score from 0 to 4: 0 = no movements, 1 = occasional movements, 2 = frequent movements, 3 = constant movements stopped by a sensory external stimulus, 4 = constant movements unstopped by a sensory external stimulus. AIMs are quantified each 20 minutes for 2h. (e.g. Cenci *et al.*, 1998; Lee *et al.*, 2000a; Lundblad *et al.*, 2002; Winkler *et al.*, 2002). The AIMs rating scale developed by M.A Cenci became the one commonly use for rodents LID rating (Cenci *et al.*, 2002). AIMs appear with therapeutic doses of L-Dopa, only when >80% of striatal DA terminals or nigral DA neurons are lost, and AIMs' severity is maximal, only when the extent of DA denervation exceeds 90% (Winkler *et al.*, 2002). The L-Dopa-induced AIMs (i) model the peak-of-dose LID, (ii) disrupt the physiological motor activities as in human LID (Lundblad *et al.*, 2002; Winkler *et al.*, 2002) and (iii) are improved by drugs such as amantadine, 5-HT_{1A} agonists and α2-adrenoreceptor antagonists as human LID (Dekundy *et al.*, 2007; Lundblad *et al.*, 2002). As for the sensitised rotational behaviour, severity of AIMs increases over time and plateaus after few days.

Although the relationship was known, Putterman et al. have carefully established that AIMs are related to dose and duration of L-Dopa treatment. In other words, given an adequate dose and magnitude of striatal DA depletion, L-Dopa produces dyskinesia with a continuous spectrum of severity. Moreover, they also showed that AIMs scores reach maximum values 60 min after L-Dopa administration (Putterman *et al.*, 2007).

While such models can highlight mechanisms responsible for motor fluctuations generally, depending upon the details of the experimental protocol employed, the studies might throw more light upon the nature of the wearing-off phenomenon (Engber *et al.*, 1989) or AIMs. (Cenci *et al.*, 1998; Henry *et al.*, 1999). However, the AIMs seen in rodents are not in a form that is obviously, and unequivocally, equivalent to chorea and dystonia seen in patients (Cenci *et al.*, 1998; Henry *et al.*, 1999). Thus, while it is likely that certain behaviours seen in 6-OHDA-lesioned rats share similar molecular, cellular and pharmacological mechanisms as LID and are thus a rodent correlate of those, it is not clear as to whether these data might be best interpreted in relation to chorea, dystonia or some other movement disorder seen in patients.

4.3.2. Mouse model

Since the systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice, although commonly used to produce degeneration of DA neurons, fails to produce consistent and stable symptoms of parkinsonism (Bezard *et al.*, 1998), the model is not in use for the study of LID. The 6-OHDA has therefore been used in a few studies to produce stable, unilateral DA lesions in mice (Akerud *et al.*, 2001; Fredduzzi *et al.*, 2002). Lundblad and co-workers have fully characterized this lesion procedure in mice, using doses of the toxin that do not produce nonspecific tissue damage (Kirik *et al.*, 1998), and injected them either in MFB or in the sensorimotor part of the striatum (Lundblad *et al.*, 2004). Both types of lesion produced a similar degree of forelimb akinesia on the contralateral side of the body. The lowest dose of L-Dopa that could significantly relieve this akinetic deficit (i.e., 6 mg/kg) did not differ between MFB and intrastriatal lesions. However, the L-Dopa threshold dose for the induction of dyskinesia did differ between the two lesion types, requiring a daily dose of 6 mg/kg for MFB lesioned mice against 18 mg/kg in the intrastriatally lesioned animals to develop abnormal movements affecting orofacial, trunk, and forelimb muscles on the side contralateral to the lesion (Lundblad *et al.*, 2004). In addition, L-Dopa-induced mice AIMs were not expressed by animals treated with ropinirole or KW-6002 at doses that improved forelimb akinesia, and were significantly reduced by the acute administration of compounds that have been shown to alleviate LID both in parkinsonian patients and in rat and monkey models of PD (e.g. amantadine, buspirone, riluzole) (Lundblad *et al.*, 2005).

The model is of great scientific value but at the cost of intense laboratory pain. The model, although behaviourally less satisfactory than its rat equivalent, offers the advantage of enabling molecular studies in genetically-modified mice. Consequently, a large number of ground-breaking papers relied upon this model (Ahmed *et al.*, 2010; Alcacer *et al.*, 2012; Cenci and Lundblad, 2007; Crittenden *et al.*, 2009; Fasano *et al.*, 2010; Fieblinger *et al.*, 2014; Francardo and Cenci, 2014; Francardo *et al.*, 2011; Marti *et al.*, 2012; Santini *et al.*, 2009a; Santini *et al.*, 2012; Santini *et al.*, 2009b; Santini *et al.*, 2010b; Santini *et al.*, 2007; Valjent *et al.*, 2005). This is however at the expense of intense animal care that regrettably does not prevent the model to suffer from a high mortality rate. Unilateral lesion of the MFB in the mouse is indeed causing, at odds with the unilateral MFB rat model, adipsia, aphagia and anhedonia. Intense animal care with high sucrose and fat diet, glucose and saline solutions injection, warmer atmosphere cannot circumvent this issue and only experienced

laboratories have proven capable of handling it (Francardo *et al.*, 2011; Lundblad *et al.*, 2005).

4.4. Non-human primate models of LID

4.4.1. History

Modelling LID was possible in non-human primates because of the now gold standard model of parkinsonism obtained with MPTP intoxication. MPTP is a neurotoxin that causes a form of parkinsonism in humans not distinguishable from idiopathic PD (Langston *et al.*, 1983). MPTP victims show all of the problems typically encountered with L-Dopa therapy, including the wearing-off and on–off phenomena, peak-dose dyskinesia and psychiatric complications (Langston and Ballard, 1984). MPTP lesioned non-human primates chronically treated with L-Dopa display the most human-like symptoms of LID. They carry on the two main criteria needed to induce LID: a loss of the nigral DAergic neurons, the neuronal population mostly involved in PD (but not the only one) and the ability to express dyskinesia after a chronic L-Dopa treatment (Iderberg *et al.*, 2012).

MPTP discovery in 1982 relies on a group of young drug addicts displaying a clinical profile almost indistinguishable from PD after the self-administration of a synthetic heroin analogue contaminated by MPTP. The symptom analogy was so closed to PD that MPTP was quickly administrated to different animal models and was able to reproduce most of the clinical and pathological hallmarks of PD in non human primates (Chiueh *et al.*, 1984; Crossman *et al.*, 1985; Doudet *et al.*, 1985; Langston *et al.*, 1984b). The MPTP discovery as a cause of parkinsonism has led to the development of valuable experimental models of PD in non-human primates (Bédard *et al.*, 1992; Bezard *et al.*, 1998; Langston *et al.*, 2000). Intracarotidian or systemic MPTP intoxication induce a degeneration of the DArgic neurons residing in the substantia nigra pars compacta leading to a DA depletion in the caudate-putamen in similarity to PD (Bezard *et al.*, 2001d; Burns *et al.*, 1983; Engeln *et al.*, 2014; Guigoni *et al.*, 2005c; Jan *et al.*, 2003; Jenner *et al.*, 1984). Lesions of the serotoninergic and noradrenergic systems, as in PD, may also be affected (Engeln *et al.*, 2014; Pifl *et al.*, 1991; Rylander *et al.*, 2010b). MPTP intoxication produces a parkinsonian syndrome in primates that is remarkably similar to PD (Benazzouz *et al.*, 1992; Bezard *et al.*, 1998). The animals display bradykinesia, rigidity and postural abnormalities (e.g. Bédard *et al.*, 1992; Benazzouz

et al., 1992; Bezard and Przedborski, 2011; Langston *et al.*, 1984a; Langston *et al.*, 2000; Schultz *et al.*, 1985) and in some cases/species resting tremor (François *et al.*, 1998). Those symptoms respond positively to the medications available in the clinic (Cenci *et al.*, 2002; Fox and Brotchie, 2010). A number of DAergic (Iderberg *et al.*, 2012; Meissner *et al.*, 2011) or surgical (Benazzouz *et al.*, 1993; Jarraya *et al.*, 2009; Kordower *et al.*, 2006) therapies have been investigated in MPTP-treated primates and subsequently successfully transferred in clinical practice. So far, effects observed in MPTP-treated primates have proven to be predictive of symptomatic efficacy in human, provided the magnitude of the effects in this model was large enough for overcoming the inherent disease and human variability. L-Dopa treatment to such MPTP-lesioned primates causes dyskinetic motor manifestations that are remarkably similar to those displayed by patients; Chorea and dystonia are easily distinguished and clearly equivalent to their human counterparts (for review, see Fox *et al.*, 2012; Langston *et al.*, 2000).

Four species have been regularly used, namely the macaque monkeys (*macaca mulatta* and *macaca fascicularis*), the common marmoset (*callithrix jacchus*) and the squirrel (S*aimiri sciureus*) monkeys, while other species might appear in the literature such as baboon monkeys (papio anubis) and African green monkeys (cercopithecus aethiops). The vast majority of pathophysiological studies has however been conducted in the macaque monkeys.

4.4.2. Macaques

The cynomolgus (*macaca fascicularis*), rhesus (*macaca mulatta*) and Japanese (*macaca fuscata*) macaques display the human-like symptoms of PD and LID and are currently the species of choice to study LID (Bezard *et al.*, 2001b; Iderberg *et al.*, 2012; Jenner, 2003b; Langston *et al.*, 2000; Morin *et al.*, 2013; Porras *et al.*, 2012b). Dyskinetic MPTP-intoxicated macaques exhibit various combinations of choreic-athetoid (i.e. characterized by constant writhing and jerking motions), dystonic and even ballistic movements (i.e. large-amplitude flinging, flailing movements), although less frequently for those latter (Bezard *et al.*, 2001b; Iderberg *et al.*, 2012; Jenner, 2003b; Langston *et al.*, 2000; Morin *et al.*, 2013; Porras *et al.*, 2012b). Both the repertoire and severity of dyskinesia are not distinguishable from LID occurring in PD patients (Bezard *et al.*, 2003).

Several rating scales exist to quantify LID in non human primates and were recently reviewed and criticized, with a focus on macaques (Fox *et al.*, 2012). Fox and co-workers proposed a revised LID rating scale in monkeys as well as guidelines for their observation: the Non Human Primate Dyskinesia Rating Scale (NHPDysR), based on the Dyskinesia Disability Rating Scale (Fox *et al.*, 2012). For each AIMs, the monkeys are scored from a score of 0 to 4 : 0 = no AIMs, 1 = mild LID : Transient and intermittent AIMs present of less than 30% of the scoring time , 2 = Moderate LID : Monkeys display AIMs for more than 30% of the observation period and are still able to perform all motor tasks, 3 = Marked LID : AIMs are present less than 70% of the scoring period and the monkeys are unable to eat with significant decrease in motor tasks. 4 = Severe LID : AIMs are continuous and present for more than 70% of the observation period. LID interfere with the ability to do any motor task (Fox *et al.*, 2012). The anti-parkinsonian effect of L-Dopa therapy is quantified using rating scales based on the UPDRS (Imbert *et al.*, 2000).

Once parkinsonism is stable, macaques are then treated with daily administration of levodopa (Levodopa/carbidopa, ratio 4:1) for 4-5 months at an individually-tailored dose designed to produce a full reversal of the parkinsonian condition, i.e. in a clinically-relevant approach. Over this period, animals develop consistent and reproducible dyskinesias (Iderberg *et al.*, 2012). Moreover, even if the L-Dopa treatment is stopped for months, only one L-Dopa administration will be necessary to induce the same LID as observed before (Ahmed *et al.*, 2010; Bezard *et al.*, 2003; Bezard *et al.*, 2004; Fasano *et al.*, 2010; Gold *et al.*, 2007b; Iderberg *et al.*, 2012; Porras *et al.*, 2012b; Rylander *et al.*, 2010a), i.e. a profile of response observed in humans as well who do not benefit from drug holidays. Although L-Dopa doses are individually tailored, the benefit of the L-Dopa therapy is invariable and consistent for each MPTP macaque, which mimics the treatment adaptation to parkinsonian patients performed in the clinic and underlies how this model is relevant before undergoing clinical trials.

Even if the similarity to human symptoms makes the MPTP macaque relevant on a clinical level for LID, this model is expensive, sizable (weighing up to 7-10kg) and need specialized infrastructures with highly qualified personnel to handle the animals (Iderberg *et al.*, 2012; Morin *et al.*, 2013). Moreover, neither the extent nor the pattern of nigrostriatal lesioning are sufficient to explain the occurrence of LID in the MPTP macaque model of LID (Fernagut *et al.*, 2010; Guigoni *et al.*, 2005b). In addition, chronic administration of high dose L-Dopa (80

mg/kg) for several months can provoke dyskinesia in normal macaque (Pearce *et al.*, 2001). Based upon these data, it is necessary to include in future investigations normal animals treated with L-Dopa as control, in addition to an other essential control group, L-Dopa treated animals with nigral lesion that do not develop dyskinesia (Engeln *et al.*, 2014; Fernagut *et al.*, 2010; Porras *et al.*, 2012a; Santini *et al.*, 2010a).

4.4.3. Marmoset

Common Marmosets (*callithrix jacchus*) were used because of their small size and their convenience in housing and handling compared to macaques. They feature a cerebral conserved structural organization. In the 80's, Marmoset models were developed and commonly used, mostly in the UK, due to both ethical and practical considerations. Parkinsonian marmoset models can be induced by either systemic MPTP intoxication (Jenner *et al.*, 1984) or by unilateral (Annett *et al.*, 1992) or bilateral (Mitchell and Carroll, 1997; Mitchell *et al.*, 1995) repeated intracerebral injections of 6-OHDA in the nigrostriatal bundle. Very few studies used the 6-OHDA-lesioned marmosets to analyse LID pathophysiology (Pirker *et al.*, 2001; Svenningsson *et al.*, 2002). Most studies relied upon the MPTP-treated marmoset that displays a parkinsonian state that reverses after administration of L-Dopa and other DA agonists (Jenner *et al.*, 1984). Following L-Dopa administration, MPTP-treated marmosets display dyskinetic-like abnormal involuntary behaviours including chorea-like (i.e. picking/flicking movements), dystonic-like (i.e. sustained posturing) and repetitive purposeless movements (Pearce *et al.*, 1995). MPTP-intoxicated marmosets chronically treated with L-Dopa are often restless and show consistent and continuous hyperkinectic behaviour at the L-Dopa peak-time of action (Iderberg *et al.*, 2012; Morin *et al.*, 2013; Pearce *et al.*, 1995). Moreover, the marked hyperkinesia of dyskinetic marmoset highly complexifies the distinct assessment of choreic-like and dystonic-like abnormal movements, which clearly differs from clinical observation of dyskinetic patients (Fox and Brotchie, 2010).

Kuoppamäki and co-workers reported on the relationship between L-Dopa dose and the duration and severity of dyskinesia in MPTP-treated marmoset with marked nigral degeneration mimicking late stage PD (Kuoppamaki *et al.*, 2007). With increasing doses of L-Dopa, locomotor activity increased and motor disability declined. The duration of dyskinesia following L-Dopa administration dose-dependently increased and showed a linear correlation

with total locomotor activity (Kuoppamaki *et al.*, 2007). In contrast, severity of dyskinesia showed a nonlinear correlation with total locomotor activity, low doses of L-Dopa eliciting severe dyskinesia for short periods of time (Kuoppamaki *et al.*, 2007).

The use of the MPTP-treated marmoset model to study novel therapies for LID was facilitated by its property to respond with a strong motor activity to L-Dopa therapy (Iderberg *et al.*, 2012), allowing to easily quantify the anti-parkinsonian state and the prototypical development of LID in response to a give test item (Hill *et al.*, 2004; Huot *et al.*, 2011; Kobylecki *et al.*, 2011; Maratos *et al.*, 2001; Pearce *et al.*, 1998). Such *de novo* protocol remains an asset of the model (Nash *et al.*, 2000) as the kinetics of LID appearance in this model is far less variable than in macaque (unless macaques are left without dopamimetic therapy for months before starting such *de novo* protocol). However, the difficulty in distinguishing choreic-like movements from dystonic-like ones during LID is not representative of the human LID pattern of expression and represent a drawback for this model to understand LID pathophysiology (Iderberg *et al.*, 2012; Morin *et al.*, 2013). Finally, while the marmoset model has been heavily used in the 90's, the development of experimental research in macaques, making them relatively easily available, has led the field away from this species, as attested by the decreasing number of papers relying on this species in the past 5 years.

4.4.4. Squirrel monkeys

As for the common Marmoset, squirrel monkeys (S*aimiri sciureus*) were employed for their convenience in handling and housing because of their small size. Squirrel monkeys intoxicated by MPTP develop parkinsonian-like syndromes as akinesia, rigidity and bradykinesia (Langston *et al.*, 1984a). MPTP-lesioned squirrel monkeys treated with L-Dopa display dyskinetic-like movements including a choreic and dystonic component (Boyce *et al.*, 1990b; Di Monte *et al.*, 2000). However, chorea is always most prevalent at the L-Dopa peak-time of effect while dystonia is barely noticeable (Boyce *et al.*, 1990b). The ability of squirrel monkeys to display dyskinesia allowed several investigations on LID pathophysiology using this model (Boyce *et al.*, 1990a; Boyce *et al.*, 1990b; Di Monte *et al.*, 2000; Hsu *et al.*, 2004; Neale *et al.*, 1984; Stephenson *et al.*, 2005) with a pronounced focus on the opioid (Chen *et al.*, 2005; Cox *et al.*, 2007; Quik *et al.*, 2002a) and nicotinic system (Quik *et al.*, 2003; Quik

et al., 2007a; Quik *et al.*, 2013c; Quik *et al.*, 2002b; Quik *et al.*, 2005; Zhang *et al.*, 2013). However, Togasaki and co-workers demonstrated that normal squirrel monkeys (i.e. unlesioned) treated twice daily with a therapeutically relevant dose of L-Dopa (15mg/kg with carbidopa, *per os*) for 15 days can develop LID (Togasaki *et al.*, 2005; Togasaki *et al.*, 2001). Even if this finding limits the translational value of the MPTP-intoxicated squirrel monkey in LID, it should very interesting to understand the physiological and the molecular basis on how L-Dopa can induce dyskinesia without any nigrostriatal denervation.

4.4.5. Other species

Other non-human primate species are used to investigate PD and LID pathophysiology such as the baboons (*papio anubis*) or the African green monkeys (*cercopithecus aethiops*).

African green monkeys can be qualified as mid-sized non-human primate weighing around 3 to 7 kg at adulthood requiring specific infrastructure, just like macaques. MPTP intoxication of African green monkeys is able to induce parkinsonian-like symptoms including akinesia, bradykinesia, rigidity and tremor (Elsworth *et al.*, 1987, 1990; Taylor *et al.*, 1994, 1997; Wichmann *et al.*, 1999). Therefore, since macaques seldom present tremor once lesioned, African green monkeys have been used to study parkinsonian tremor besides other aspects of PD pathophysiology (Bergman *et al.*, 1998; Boulet *et al.*, 2008; Guehl *et al.*, 2003; Mounayar *et al.*, 2007; Pessiglione *et al.*, 2003; Rosin *et al.*, 2011; Wichmann *et al.*, 1999). Only a few studies focused on LID pathophysiology (Heimer *et al.*, 2002; Heimer *et al.*, 2006) or risk for developing LID in response to transplantation of fetal dopaminergic neurons treatment (Redmond *et al.*, 2008).

Baboons are sizable non-human primates weighing up to 40kg at adulthood and require particularly suitable infrastructure and highly qualified personnel to handle the animals. Only few research teams still work with them in the world. MPTP-intoxicated baboons develop PD symptoms such as hypokinesia, bradykinesia, postural impairments, rigidity and resting tremor (Hantraye *et al.*, 1993; Varastet *et al.*, 1994). They were used to mostly investigate PD pathophysiology and innovative surgical therapeutic approaches because of their brain size (Chen *et al.*, 2008; Drouot *et al.*, 2004; Ferrante *et al.*, 1999; Hantraye *et al.*, 1996; Kishima *et al.*, 2004; Todd *et al.*, 1996). Although not directly used for the pathophysiology of LID as

chronic oral treatment in such animals is far too risky, there are physiologically capable of displaying abnormal involuntary movements. Baboons were indeed used to model Huntington's disease (Hantraye *et al.*, 1990; Isacson *et al.*, 1989; Palfi *et al.*, 1996). For instance, excitotoxic striatal lesions with 3-nitropropionic acid induces dyskinetic-like abnormal movements following apomorphine injection (Palfi *et al.*, 1997).

4.5. Models of DDS/ICD

Increased awareness about the dramatic consequences of DDS and ICD for PD patients (Giovannoni *et al.*, 2000; Lawrence *et al.*, 2003; Voon *et al.*, 2011b; Weintraub *et al.*, 2010), prompted to investigate their underlying mechanisms. Imaging studies (Evans *et al.*, 2006; Thobois *et al.*, 2010; van Eimeren *et al.*, 2010) as well as impulsivity evaluation (Djamshidian *et al.*, 2011a; van Eimeren *et al.*, 2009; Voon *et al.*, 2007b) have provided insights into some of the alterations underlying these troubles and regarding associated risk factors. However, these studies being mainly conducted in PD patients already under DAergic treatments and usually with documented DDS or ICD histories, it is virtually impossible to decipher the respective contributions of the degenerative process, DRT, and individual vulnerability factors.

The progressive DAergic loss occurring in both nigrostriatal and mesolimbic pathways and the subsequent action of DRT on these altered pathways have been proposed to disrupt the reward system (Ambermoon *et al.*, 2011; Giovannoni *et al.*, 2000; Lawrence *et al.*, 2003). Indeed, by many aspects DDS as well as ICD remind addiction to drug of abuse and behaviors associated with the abuse of psychostimulants. Multiple works thus proposed that, in addition to the data coming from experiments on basal ganglia dysfunction in PD, the addiction framework might help to understand and to study these non-motor side-effects (Ambermoon *et al.*, 2012; Ambermoon *et al.*, 2011; Bearn *et al.*, 2004; Giovannoni *et al.*, 2000; Lawrence *et al.*, 2003). The study of ICD and DDS might thus take advantage of works conducted in both the PD and the addiction field. While there is a great diversity of experiments to test addiction theories in animals, there are currently no distinct DDS or ICD animal models as for LID. However, multiple aspects involved in the emergence of DDS or ICD can be reproduced in animal and give information on their triggering factors that might not be directly assessed in patients:

4.5.1. The role of the DAergic medication

An action of DRT on reward pathways has been proposed to disturb reward processing (Evans *et al.*, 2010). To measure the effectiveness of DRT to act on these pathways, experiments conducted in rodents aimed to evaluate both reward processing disruption and reinforcing properties of DRT. The DAergic D2/3 receptor agonists have been mainly incriminated in ICD in human (Gallagher *et al.*, 2007; Weintraub *et al.*, 2010). Using various operant conditioning paradigms, experiments on rats evaluated the impact of Pramipexole (PPX) on impulsive behaviors and revealed that naive rats dose-dependently do more impulsive choices under PPX, preferring smaller-sooner over larger-later rewards (Koffarnus *et al.*, 2011; Madden *et al.*, 2010). Such results are consistent with reports of parkinsonian patients doing more impulsive choices and being more sensitive to delay under DA agonists (Voon *et al.*, 2010). Other works evaluating the effect of PPX on risk taking in rats using operant tasks allowing the choice between a safe option providing a small reward and a more hazardous option offering a larger reward showed that under PPX, animals dose-dependently increase their choice toward the most risky option (Johnson *et al.*, 2011; Johnson *et al.*, 2012). These results are reminiscent of the described desensitization to punishment and increased risk taking of PD patients with pathological gambling under DA agonists (Djamshidian *et al.*, 2010; Voon *et al.*, 2011a; Voon *et al.*, 2007b). Finally, some studies evaluated the ability of D2/3 agonists to act on the reward pathway as hypothesized for ICD (Gschwandtner *et al.*, 2001; Schott *et al.*, 2007). In self-administration procedure, D2/3 agonists have been shown to dose-dependently maintain responding to stimuli previously paired with cocaine (substitution), but also to induce responding for stimuli associated with cocaine (Collins *et al.*, 2012; Collins and Woods, 2007). Later studies further revealed that naïve, as well as parkinsonian rats display conditioned place preference (Riddle *et al.*, 2012) or can self-administer PPX (Engeln *et al.*, 2012), demonstrating the rewarding properties of D2/3 agonists. Experiments evaluating the ability of DAergic treatments to trigger ICD- or DDS-like features on naïve (e.g. non DA lesioned) animals might however bring incomplete insight on the nature of these troubles. Indeed, despite the fact that ICD are also reported in non DA-depleted individuals (Davie, 2007; Holman, 2009; Ondo and Lai, 2008), the neurodegenerative process occurring in PD has been questioned in the development of impairments in reward processing in both ICD and DDS (Lawrence *et al.*, 2003; Schott *et al.*, 2007).

4.5.2. The role of the DAergic loss

The onset of ICD or DDS with the apparition of PD raised questions on the contribution of both nigrostriatal and mesolimbic DArgic degeneration in the emergence of these side-effects of DRT (Lawrence *et al.*, 2003; Thobois *et al.*, 2010). While unilateral lesioning of mesencephalic DArgic systems is well-suited for the study of LIDs, owing to a marked DArgic asymmetry facilitating the quantification of abnormal motor behaviours, such models are not adapted for studying non-motor aspects of DRT as drug-induced rotations will bias operant behaviors. Obtaining a viable bilateral lesion model is however uneasy (Dunnett and Lelos, 2010; Ungerstedt, 1971a) and might in part contribute to the relatively small number of work conducted so far in rodent models of PD. Using different bilateral lesioning strategies, several studies have investigated how the DAergic lesion may affect behavioral dimensions relevant to ICD and DDS. Lesion studies in rats first highlighted that a bilateral nigrostriatal lesion using 6-OHDA could reduce motivation in rats in various paradigms such as place preference, instrumental responding or food consumption test (Drui *et al.*, 2013; Faure *et al.*, 2005; Pioli *et al.*, 2008). Bilateral lesions of the nigrostriatal pathway obtained by injecting 6-OHDA in the dorsal striatum showed that the establishment of a conditioned place preference for PPX necessitated a greater dose in sham than in lesioned rats (Riddle *et al.*, 2012). Moreover, risk-taking evaluation showed that PPX increased intracranial self-stimulation-mediated probabilistic discounting in both sham and lesioned rats (Rokosik and Napier, 2012). Mixed bilateral nigrostriatal and mesolimbic lesions achieved with intracerebroventricular 6-OHDA infusion however resulted in similar reinforcing properties, comparable motivation and equally low drug-seeking for PPX between sham and lesioned rats using intravenous self-administration procedures (Engeln *et al.*, 2012). Conditioned place preference experiments in animals injected with small 6-OHDA doses in the medial forebrain bundle showed that high doses of bromocriptine (DA D2 agonist) might have rewarding properties, but failed to report such results for supraliminal (50-200 mg/kg) doses of L-Dopa (Zengin-Toktas *et al.*, 2013). Recently, bilateral nigrostriatal lesions were obtained by using viral-mediated α-synuclein overexpression in the substantia nigra (Engeln *et al.*, 2013). This latter model is promising as it reproduces many of the cellular and molecular features of PD, including early synaptic dysfunction as well as accumulation and aggregation of α-synuclein (Decressac *et al.*, 2012a; Decressac *et al.*, 2012b). In these animals, a moderate lesion of the substantia nigra was necessary and sufficient to reveal psychostimulant-like properties of L-Dopa. Indeed, clinically relevant doses of L-Dopa (12 mg/kg) elicited a conditioned place

preference as well as a decreased interest for a non-drug reward exclusively in lesioned rats (Engeln *et al.*, 2013). These latter findings provided significant details on how L-Dopa might act on altered DAergic pathway to result in reward dysfunction as observed in DDS.

In human, the role of the DAergic loss is difficult to evaluate, as it would require large prospective studies comparing patients before and after the diagnosis of PD. To date, only one study conducted in α-synuclein duplication carriers compared presymptomatic and symptomatic stages, showing reward impairments solely after the establishment of significant neurodegeneration (Szamosi *et al.*, 2013).

It is interesting to notice that in rodents, DA agonists may trigger addiction-like behavior in both control and lesioned rats, while L-Dopa is rewarding only in lesioned animals. Such observations are in agreement with clinical reports of low mood elevation effects of L-Dopa in normal individuals (Liggins *et al.*, 2012). Thus, after evaluating the potential for DArgic treatments to act on the reward pathway of lesioned animals, studies are now evaluating their ability to affect impulsive behaviors.

Finally, clinical studies have suggested that individual risk factors could contribute to the emergence of both ICD and DDS (Djamshidian *et al.*, 2011b; Evans *et al.*, 2005; Voon *et al.*, 2007b). A family history of gambling, a higher alcohol consumption or previous use of legal or illegal drugs is associated with both increased DDS and ICD incidence (Evans *et al.*, 2005; Weintraub *et al.*, 2010). Similarly, personality traits such as sensation seeking, impulsiveness or risk taking might be triggering elements (Voon *et al.*, 2011b). These traits might be present before PD's motor symptoms and could also evolve with the progression of the disease process. While a recent study suggested that unmedicated *de novo* PD patients have no increased risk to develop ICD compared to the general population (Weintraub *et al.*, 2013), it is unknown if the ongoing degenerative process may affect impulsiveness in these patients. Experimental models will help to solve this question.

4.5.3. The role of individual risk factors

The most difficult aspect of ICD and DDS to investigate in human is the influence of the DAergic depletion on personality traits. Information on premorbid 'baseline' impulsive

behaviors obtained retrospectively by interviewing patients or their family might be biased and misleading. Surprisingly, there is currently no animal study providing a longitudinal measurement of impulsive behaviors before and after DAergic depletion. A prelesional screening of impulsiveness might yet be crucial in the understanding of ICD pathophysiology. Behavioral tests such as delay discounting tasks, evaluation of impulsive choices and actions, risk taking measures are available in rodents and could provide important information on the evolution of the personality trait over the course of the disease. Thus, prelesional vs. postlesional inhibitory control assessment would help to elucidate if and how the DAergic denervation may affect these individual traits. Moreover, the differential impact of DRT on behavioral inhibition according to specific behavioral traits would help to assess individual responses to the treatment of PD. Future experiments in this direction will be mandatory as individual vulnerability appears to be a critical factor in the propensity to develop non-motor side effects of DRT.

Rodent models of ICD and DDS offer the possibility to measure cellular and molecular changes occurring after lesion, drug exposure and behavioral tasks. Again, only few data are currently available regarding these factors. Experiments evaluating the reinforcing properties of D2/3 agonists in rats revealed that PPX induces striatal molecular changes (Engeln *et al.*, 2012). Moreover, motivation for PPX was correlated with molecular markers in different striatal compartments in sham and lesioned rats, suggesting the involvement of different cortico-subcortical loops in DA depleted animals. Interestingly, a modified expression of molecular markers was found in the dorsal striatum of lesioned rats, a region mainly described for its role in dyskinesia (Cenci *et al.*, 1999). In psychostimulant addiction, drug seeking (Yin *et al.*, 2005) and habit learning (Yin *et al.*, 2004) are known to involve dorsal regions of the striatum. Altogether, these observations suggest that common mechanisms may operate in motor and non-motor side-effects of DRT where specific molecular modifications underlie both motor and reward dysfunctions. Indeed, similarities between the repetitive movements of dyskinesia and the stereotyped behaviors of punding are observed. It has been proposed that both dyskinesia and ICD or DDS might be part of the same continuum (Voon *et al.*, 2009). In rats, procedures used to induce stereotypies are comparable to those used to induce dyskinesia, and these two behaviors induce similar striatal molecular adaptations (Graybiel *et al.*, 2000). Moreover, according to the striosome/matrix theory of striatal functioning, while stereotypies induce molecular changes in the matrix of the medial striatum, LID induce modifications in the matrix of the lateral striatum. In susceptible individuals, the

activation of striosomes at the interface between the 'limbic' medial striatum and the 'motor' lateral striatum might underlie compulsive behaviors such as punding (Graybiel *et al.*, 2000). Studies conducted in DA depleted rats further showed that an increase in the DAergic tone leads to a global involvement of the striatum and could jointly activate neuronal networks which were previously distinct (Saka *et al.*, 1999). It is thus possible that the compulsive/addiction-like behaviors and dyskinesia may share similar neuronal circuits. Considering the limbic and motor interfaces of the striato-nigro-striatal spiraling pathways (Haber *et al.*, 2000), a molecular sensitization of the connectivity between the ventral and the dorsal striatum might operate both in motor and non-motor side-effects of DRT. Studies investigating the possible common pathways of both LID and compulsive behaviors might provide new opportunities to approach broad basal ganglia modification linked with DRT.

In summary, experimental modelling of DDS and ICD is still in its infancy and there is currently no validated animal model replicating the clinical features of these non-motor side effects of DRT. However, works conducted in rodents already provided precious information on the respective and combined roles of the DRT and the DAergic depletion in triggering ICD- and DDS-like features. Further studies are now required to understand the role and the evolution of personality trait over the course of the lesional process. Upcoming studies will also need to provide information regarding the cellular and molecular changes occurring in these troubles. It has to be noticed however that animal studies might carry some limitations. While LIDs may affect virtually all PD patients over chronic exposure, DDS occur in 1-4% and ICD are reported in 6 to 14% of PD patients (Giovannoni *et al.*, 2000; Weintraub *et al.*, 2010). Assuming that such prevalence would be reproduced in animals, large numbers of subjects will be required. Screenings of susceptible animals (Deroche-Gamonet *et al.*, 2004; Lenoir *et al.*, 2013) might however help to overcome these limitations. Dimensions of ICD and DDS, such as financial loss, are difficult to model in rodents and could participate to experimental limitations. The use of primate models would be of great interest to study higher cognitive processes.

4.6. Modelling other L-Dopa-induced side-effects

Autonomic and psychiatric side-effects of L-Dopa have received very little attention in experimental models of PD. Even though several models recapitulate some of the

cardiovascular features of PD, including depletion of norepinephrine and reduced Meta-iodobenzylguanidine (MIBG) uptake (for review , see Fleming, 2011), the effects of L-Dopa on cardiac function have not been examined in experimental models. There is however evidence in normal rats that L-Dopa can affect the baroreflex and, interestingly, such effect may be related to a direct L-Dopaergic action in the nucleus of the solitary tract (Kubo *et al.*, 1992; Misu *et al.*, 1995; Yue *et al.*, 1994).

The effects of L-Dopa on vigilance (excessive daytime sleepiness, sleep attacks) in experimental models of PD remain poorly known. In MPTP-treated monkeys, L-Dopa did not affect rest-activity rhythms (Vezoli *et al.*, 2011). On the other hand, a high-dose of L-Dopa (50 mg/kg) was found to increase wakefulness and to decrease slow wave sleep and paradoxical sleep in MPTP-treated mice (Laloux *et al.*, 2008).

Few studies have investigated supposed "psychosis-like" behaviours in experimental models f PD and have assessed the effects of DA replacement therapy, including L-Dopa (Johnston *et al.*, 2011; Visanji *et al.*, 2006). Whether the observed behaviours (stereotypies, agitation/hyperactivity, repetitive grooming, staring or tracking an apparent non-stimulus) may represent adequate correlates of psychotic behaviours occurring in PD remain hypothetical. Indeed "psychosis-like" behaviours systematically occur in all L-Dopa-treated animals (Johnston *et al.*, 2011; Visanji *et al.*, 2006), while such behaviours are only observed in a subset of PD patients. Given the multifactorial nature of psychosis in PD, including the described occurrence of hallucinations and other psychotic behaviors unrelated to L-Dopa treatment (Fenelon *et al.*, 2006), the relevance of experimental models to this aspect of PD remains difficult to ascertain.

5. Pathophysiology of Peak of Dose LID

In the present review, we aim at focusing on changes observed at the peak of dose of L-Dopa action. Indeed, in the literature, LID pathophysiology refers to various states. In several papers, animals are considered as "dyskinetic" (i.e. since they have been chronically exposed to L-Dopa) but they were actually terminated OFF L-Dopa (i.e. more than 3 hours after their last L-Dopa injection). While the OFF state is very interesting and informative on the neuronal plasticity induced by the chronic treatment, it could not be considered as the ON

LID state. Indeed, the ON LID state reflects the neuronal pathological events occurring at the peak of dose of the treatment, at which dyskinesia are the most strongly expressed, and allows a correlation between the progressive L-Dopa induced motor response and the cellular alterations. We will therefore structure this part by clearly distinguishing: naïve animals (i.e. never exposed to dopamimetics), the ON state (i.e. peak of dose of L-Dopa, best antiparkinsonian effect) with or without LID, and the OFF state (i.e. animals otherwise dyskinetic when challenged).

5.1. Pharmacokinetics and pharmacodynamics

Chronic L-Dopa administration remains the best treatment for PD since its introduction in the 60's (Birkmayer and Hornykiewicz, 1961, 1962; Cotzias *et al.*, 1967; Lees, 1994; Yahr *et al.*, 1968). However, L-Dopa therapy faces several challenges resulting from the complex interactions between the pharmacokinetics of L-Dopa itself and the progressive neuronal alterations induced by the neurodegeneration in PD. L-Dopa indeed displays particular peripheral characteristics (Contin and Martinelli, 2010; Contin *et al.*, 1993). First, L-Dopa is highly metabolized into DA by peripheral L-amino acid decarboxylase (AADC) expressed in the gut allowing only 30% of L-Dopa to reach the systemic circulation (Contin and Martinelli, 2010). This issue was overcome by a concomitant administration of AADC peripheral inhibitors (AADCI) with levodopa. Nowadays, 2 mains AADCI are used: carbidopa at a L-Dopa/carbidopa dose ratio of 4/1 and 10/1 or benserazide (L-Dopa/benserazide 4/1) (Contin and Martinelli, 2010). The used of AADCI allowed to almost tripled L-Dopa oral bioavailability, strongly reducing the required L-Dopa therapeutic dose (Contin and Martinelli, 2010). Interestingly, concomitant administration of L-Dopa and AADCI induce a plasmatic peak in patient at 1.1 ± 0.21h (Okereke *et al.*, 2004) and in non-human primate, like macaque, at 1.6 ± 0.3h (Huot *et al.*, 2012) associated with a brain Cmax which correlates with the peak of LID severity, around 60-90 minutes *post* L-Dopa uptake in macaque (Huot *et al.*, 2012). In addition, L-Dopa shares some similarities with neutral amino acids, notably in term of intestinal absorption and transport at the blood brain barrier (Nutt and Fellman, 1984). Indeed, L-Dopa seems to compete with the transport system of neutral amino acids during their absorption in the intestinal mucosa, markedly reducing the L-Dopa plasmatic concentration (Contin and Martinelli, 2010; Contin *et al.*, 1993). Moreover, L-Dopa transport from the plasma to the brain depends on the same system used in the intestine (Contin and

Martinelli, 2010). Studies displayed that an intake of high protein meals double the plasmatic concentration of neutral amino acids which interferes with L-Dopa transport to the brain and decreases its therapeutic effect (Nutt *et al.*, 1984), uncovering a competition between L-Dopa and neutral amino acids at the blood brain barrier (Leenders *et al.*, 1986). Consequently, L-Dopa administration timing needs to be adapted to mealtime to optimize the therapeutic effect of L-Dopa (Juncos *et al.*, 1987).

5.2. Neurochemistry

LIDs are associated with several modifications of neurotransmitter release. Indeed, numerous studies have reported preclinical data in animal models showing that drugs targeting diverse neurotransmitter systems can modify peak-dose LID (Huot *et al.*, 2013). We can evoke the benefit of opioidergic, serotonergic, noradrenergic, DArgic or glutamatergic drugs against peak-dose LID in animals. This suggests an aberrant increase in the neurotransmitter induced by L-Dopa in case of antagonist administration, but there are also molecular and cellular interactions of an antagonist that could explain an efficacy independently of changes in neurotransmitter release. For instance, non-selective 5-HT antagonists such as mianserin or mirtazapine may reduce LID in a situation in which 5-HT release is likely reduced or not affected during the peak-dose LID (Lindgren *et al.*, 2010; Navailles *et al.*, 2011a). In a similar way, it has been consistently shown that L-Dopa or DA agonists inhibit acutely and chronically the electrical activity of subthalamic neurons (Lafreniere-Roula *et al.*, 2010; Levy *et al.*, 2002; Lozano *et al.*, 2000; Ni *et al.*, 2001), the sole source of glutamate of the substantia nigra or the globus pallidus (Albin *et al.*, 1989a). It is therefore interesting to note that L-Dopa enhances glutamate release in the substantia nigra of dyskinetic rats (Mela *et al.*, 2012; Mela *et al.*, 2007), a finding that corroborates the clinical and preclinical evidence that glutamatergic antagonists are able to alleviate peak-dose LIDs (Bibbiani *et al.*, 2005; Duty, 2012; Verhagen Metman *et al.*, 1998b). It is therefore important to focus on the changes of neurotransmitter release in order to understand the physiopathology of LID in terms of extracellular neurotransmitters imbalance. Unfortunately, it is difficult to build a valid hypothesis regarding neurotransmitter imbalance due to the few data specifically looking at the peak-dose LID. Indeed, measuring extracellular levels of neurotransmitter is extremely difficult and the results obtained are not in the timeframe of the AIMs expression.

5.2.1. L-Dopa-induced DA release and LID: a role for the striatum ?

It has been thought for a long time that an excessive DA tone produced by L-Dopa is responsible for the occurrence of peak-dose LID. Clinically, LIDs are reduced by a variety of antipsychotic drugs, drugs that are known to block DA receptors (Durif *et al.*, 2004). The use of DA antagonists is limited due to high risk to aggravate the motor score and promoting Parkinsonism (Hagan *et al.*, 1997). In addition, the peak-dose dyskinesia is sensitive to the dose of L-Dopa, the reduction of a dose often leading to a reduction of LID (Lindgren *et al.*, 2007; Putterman *et al.*, 2007). In patients with LID, the use of PET-scan allows following caudate-putamen binding of [^{11}C]-raclopride, a DA radiotracer able to bind D2/D3 receptors. It has been consistently reported that [^{11}C]-raclopride binding in the striatum was dramatically reduced (by more than 80%) by L-Dopa administration compared to the binding obtained before L-Dopa administration (Tedroff *et al.*, 1996). The dose administered to patients corresponded to 3 mg/kg i.v. and the ability of L-Dopa to displace [^{11}C]raclopride was attributed to the increase in synaptic DA extracellular levels induced by L-Dopa (de La Fuente-Fernandez *et al.*, 2001a; de la Fuente-Fernandez *et al.*, 2001b; de la Fuente-Fernandez *et al.*, 2004a; Tedroff *et al.*, 1996). Interestingly, the decrease in [^{11}C]raclopride 1h after the oral administration of 250/25 mg of L-Dopa/carbidopa was stronger in patients with LID compared to stable responders without LID (de la Fuente-Fernandez *et al.*, 2004b). The difference was no longer observed 4h after L-Dopa administration. The displacement of [^{11}C]raclopride binding by L-Dopa at 1h was 16-17% in dyskinetic and 7-9% in non-dyskinetic patients. Thus it has been postulated that LIDs develop as a consequence of abnormal fluctuations in synaptic DA levels induced by oral L-Dopa treatment and the swings in synaptic DA would be dramatically greater in patients with dyskinesia (Cenci, 2007a; Cenci and Lundblad, 2006; de la Fuente-Fernandez *et al.*, 2004b; Olanow and Obeso, 2000).

In contrast to the initial prediction that DA synaptic concentration raised by L-Dopa would be far beyond normal levels (Tedroff *et al.*, 1996), de La Fuente-Fernandez et al clearly indicated, based on their models (de la Fuente-Fernandez *et al.*, 2001b; de la Fuente-Fernandez *et al.*, 2004a), that extracellular levels of endogenous and exogenously derived DA in Parkinsonian patients are lower, even in dyskinetic patients, compared to normal situations. However, small differences were reported in this study and should be interpreted with caution for several reasons. First, the possible head movements of patients with LID during PET-scan and the different medical history of patients (i.e., onset and duration of the disease, different

L-Dopa doses administered daily between dyskinetic and non-dyskinetic patients) can be confounding factors. Secondly, the measurement only relies on the radiotracer binding of D2/D3 receptors although these receptors can display distinct levels of activity as recently exemplified in macaques (Koprich *et al.*, 2013). Consequently, raclopride binding could be lowered by mechanisms other than an increase in synaptic DA because other neurotransmitters could affect the level of activity of D2/D3 receptors. Finally, clinical studies, while using indirect method to measure DA in humans, interpret the changes of [^{11}C]raclopride binding as the result of changes of DA released through compensatory mechanisms by spared DA terminals in the striatum (Sossi *et al.*, 2004). However, DA turnover measured by using [^{11}C]raclopride enhances as the disease progresses in humans (Rajput *et al.*, 2004). Data in animals also report a drastic change of DA metabolism after lesion of DA neurons, in line with the fact that DA is preferentially metabolized by monoamine oxydase B in lesioned rats instead of monoamine A in naïve rats (Wachtel and Abercrombie, 1994). Thus, the changes of DA turnover may not be due to drastic changes of striatal DA terminals, but to the overcoming contribution of other systems to release the newly synthesized DA in an abnormal manner as the degeneration of DA neurons progresses (see below).

The link between LID and striatal DA has been studied in different animal models but mostly in rats (Cenci *et al.*, 1998; Cenci and Lundblad, 2007; Cenci and Ohlin, 2009). In dyskinetic rats, it has been confirmed that the therapeutic efficacy of L-Dopa treatment decreases over time with the development of LIDs (Cenci and Lundblad, 2007). Using concomitant intracerebral microdialysis or in vivo amperometry, studies have shown that the role of striatal DA in LID is not that clear (Cenci and Lundblad, 2006). Indeed, L-Dopa-induced DA extracellular levels in the striatum of dyskinetic rats was either enhanced (Lundblad *et al.*, 2009), not affected (Lindgren *et al.*, 2010), or slightly decreased (Nevalainen *et al.*, 2011) compared to non-dyskinetic rats. As postulated in humans (de la Fuente-Fernandez, 2013; de la Fuente-Fernandez *et al.*, 2004b), LID can occur in rats at doses of L-Dopa (3 mg/kg; comparable to humans) whose effects on striatal DA levels are far from reaching physiological values obtained in naïve rats (**Figure 2**) (Navailles *et al.*, 2011a; Navailles and De Deurwaerdere, 2012b).

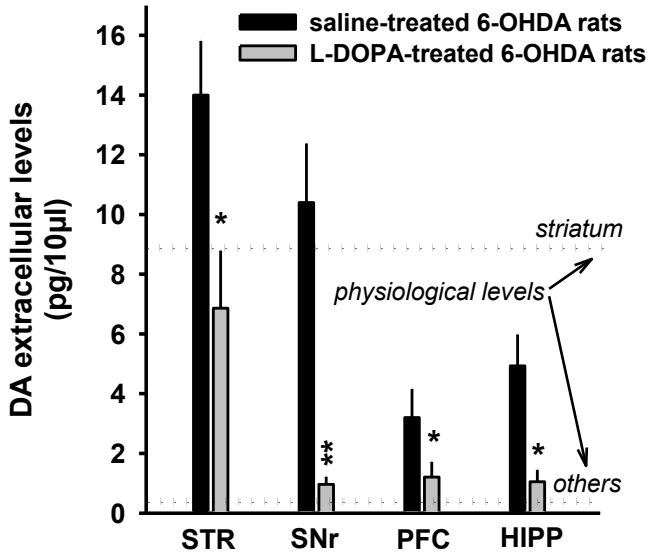

Figure 2. Effect of L-Dopa (12 mg/kg) on dopamine (DA) release in the striatum (STR), substantia nigra pars reticulata (SNr), prefrontal cortex (PFC) and hippocampus (HIPP) of 6-hydroxydopamine (6-OHDA) rats treated chronically with saline followed by one injection of L-Dopa (black bars) or L-Dopa (grey bars; 12 mg/kg/day for 10 days). Dashed lines indicate the basal extracellular levels of DA measured in brain regions of naïve rats. Two important results are found in L-Dopa-treated 6-OHDA rats that develop dyskinesia: 1) DA extracellular levels fail to reach physiological values in the striatum but are far above them in other brain regions; 2) L-DOPA-induced DA release, while mostly preserved in the striatum, is dramatically reduced in other brain regions. Data are the mean ± SEM (n = 7-8 rats/group) of the average of DA levels over three hours monitoring after L-DOPA administration. *p<0.05, **p<0.01 versus the saline-treated group (Student's *t*-test). Adapted from Navailles et al 2011a.

5.2.2. Ins and outs of L-DOPA effects on serotoninergic neurons

After the demonstration that 5-HT neurons play a pivotal role in the mechanism of action of L-Dopa, a second neurochemical hypothesis has emerged and postulates that LIDs develop as a consequence of the dysregulated release of DA as a "false neurotransmitter" from 5-HT neurons (Carta *et al.*, 2007; Lindgren *et al.*, 2010; Munoz *et al.*, 2009; Navailles *et al.*, 2010a; Navailles *et al.*, 2010b; Nevalainen *et al.*, 2011; Ulusoy *et al.*, 2010). Indeed, the inhibition of L-Dopa-induced DA release by 5-HT_1 autoreceptors stimulation (Kannari *et al.*, 2001; Lindgren *et al.*, 2010; Nahimi *et al.*, 2012) and/or 5,7-Dihydroxytryptamine (DHT) lesion of 5-HT neurons (Navailles *et al.*, 2010b; Nevalainen *et al.*, 2011; Tanaka *et al.*, 1999) is associated with a marked reduction in LIDs (Carta *et al.*, 2007; Lindgren *et al.*, 2010; Nahimi *et al.*, 2012). Still, these mechanisms were mostly described in the striatum. However, with regard to the widespread 5-HT innervation in the brain (Azmitia and Segal, 1978), other brain regions could be involved in the development of LIDs (Bastide *et al.*, 2014; Cenci and Lundblad, 2006; Di Matteo *et al.*, 2008; Marin *et al.*, 2009; Munoz *et al.*, 2009; Orosz and Bennett, 1992; Sarre *et al.*, 1992; Sarre *et al.*, 1997). Moreover, L-Dopa, by entering 5-HT neurons, mediates numerous changes in 5-HT neuron homeostasis (Navailles *et al.*, 2011a; Navailles *et al.*, 2011b; Navailles and De Deurwaerdere, 2012a, b) that could participate in the development of LIDs (Fox *et al.*, 2009; Scholtissen *et al.*, 2006). Therefore, a third hypothesis must be postulated: LIDs develop as a consequence of the imbalance in DA and 5-HT transmissions between cortical and subcortical brain regions (Navailles and De Deurwaerdere, 2012b).

5.2.2.1. The presynaptic DA effects of L-Dopa are mediated by heterogeneous 5-HT terminals

5-HT neurons possess the molecular features required for releasing DA from exogenous L-Dopa. They express the AADC that converts L-Dopa into DA and the vesicular membrane transporter VMAT2 that packages DA into exocytosis vesicles (Arai *et al.*, 1995; Ng *et al.*, 1970a; Tison *et al.*, 1991; Yamada *et al.*, 2007). Consequently, 5-HT neurons release the newly synthesized DA in a TTX- and reserpine-sensitive manner, but in a DA drug-insensitive manner (Lindgren *et al.*, 2010; Maeda *et al.*, 1999; Miller and Abercrombie, 1999). The lesion of 5-HT neurons by the selective neurotoxin 5,7-DHT drastically reduces

the increase in DA extracellular levels induced by a wide range of L-Dopa doses (3-100 mg/kg) (Navailles *et al.*, 2010b; Nevalainen *et al.*, 2011; Tanaka *et al.*, 1999). This effect is dependent on the extent of 5-HT denervation (Navailles *et al.*, 2010b), which excludes the involvement of any other cellular system in the release of DA induced by L-Dopa. Consistently, L-Dopa-induced DA release is sensitive to 5-HT auto-regulatory mechanisms. Both the stimulation of 5-HT_{1A} autoreceptors by the 5-HT_{1A} agonist 8-OHDPAT (Kannari *et al.*, 2001; Nahimi *et al.*, 2012) and the blockade of 5-HT transporters (SERT) by the selective serotonergic reuptake inhibitors (SSRI) fluoxetine (Yamato *et al.*, 2001) or citalopram (Navailles *et al.*, 2010b) reduce the increase in L-Dopa-derived DA extracellular levels. These effects are thought to occur via the inhibition of 5-HT neuron activity (Adell *et al.*, 1993; Arborelius *et al.*, 1995; Bosker *et al.*, 1996; Casanovas and Artigas, 1996; Knobelman *et al.*, 2000; Riad *et al.*, 2000; Sharp *et al.*, 1989; Sprouse and Aghajanian, 1987) and/or via an impulse-independent mechanism involving 5-HT transporters (Lindgren *et al.*, 2010; Miller and Abercrombie, 1999; Mizoguchi *et al.*, 1993; Navailles *et al.*, 2010b; Navailles *et al.*, 2011b).

Most importantly, L-Dopa induces an ectopic release of DA in a pattern that follows the widespread innervation of the entire forebrain by 5-HT raphe neurons (Azmitia and Segal, 1978). Using a multisite microdialysis approach with four probes implanted simultaneously in the ipsilateral 6-OHDA-lesioned side (Navailles *et al.*, 2013), it was shown that L-Dopa-induced DA release is not restricted to the striatum. The increase in DA release induced by an acute administration of L-Dopa also occurs in the prefrontal cortex (PFC), hippocampus (HIPP) and substantia nigra pars reticulata (SNr) (Navailles *et al.*, 2010a; Navailles *et al.*, 2010b, 2011a). This extrastriatal DA release is also sensitive to 5-HT lesion, 5-HT pharmacological manipulation and high-frequency stimulation of the subthalamic nucleus, a surgical approach in PD able to inhibit 5-HT neuronal firing (Navailles *et al.*, 2010a; Navailles *et al.*, 2010b; Temel *et al.*, 2007). All these brain regions display various levels of 5-HT terminal density and express DA receptors (Seeman, 1980) on which the newly synthesized DA can exert its numerous effects and promote DA transmission. Importantly, the magnitude of the increase in extracellular DA concentration induced by a therapeutic dose range of L-Dopa (3-12 mg/kg) is far higher in extrastriatal brain regions than in the striatum compared to the physiological situation (**Figure 2**) (Navailles *et al.*, 2010b). Therefore, the drastic increase in DA transmission in other brain regions may counterbalance the

contribution of striatal DA levels to the effects of L-Dopa in Parkinsonian conditions and prevent the occurrence of motor complications at the beginning of L-Dopa treatment.

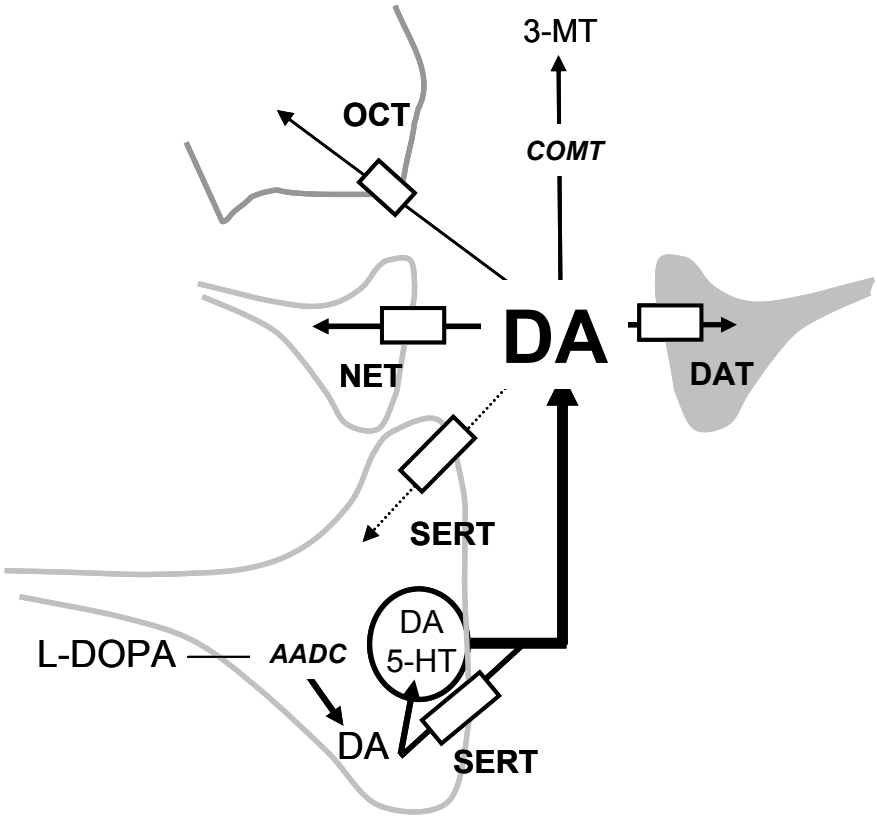

Figure 3. Drawing representing the mechanism of action of L-DOPA and the mechanisms possibly involved in the swings of L-DOPA-induced DA release. Briefly, L-DOPA enters 5-HT neurons. It is converted into DA via the l-aromatic amino acid decarboxylase (AADC). 5-HT and DA are co-stored in vesicles of exocytosis inside 5-HT neurons, and co-released. Newly synthesized DA may leak in the extracellular space via the reversal of the SERT. Extracellular DA is taken up by the DAT. The efficacy is dependent on the status and the number of spared DA terminals in the striatum and/or nucleus accumbens. In other brain regions, NA neurons take up extracellular DA via the NET and the efficacy of the clearance is dependent on spared NE terminals. Additionally, other transporters such as the organic cation transporters (OCT) or metabolic enzymes such as the catechol-O-methyl transferase (COMT) converting DA into 3-methoxytyramine (3-MT) play also a role in the clearance of extracellular DA. The SERT, to a lesser extent than other transporters, could also participate in the clearance of extracellular DA. Thus, the existence of swings of synaptic DA induced by L-DOPA may occur in numerous brain regions and their dynamic may depend on the various clearance processes operating in each brain region.

Of particular relevance, it has been described for many years that an increase in cortical DA counteracts aberrant DA signalling in subcortical areas. For instance, the catalepsy induced by the DA antagonist haloperidol, a rat model of Parkinsonism, is reversed by the direct infusion of DA into the PFC (Tucci *et al.*, 1994). Moreover, the increase in DA release induced by L-Dopa is very high in the SN, a brain region that receives the densest 5-HT innervation (Azmitia and Segal, 1978) and that directly participates in the motor effects of L-Dopa in the 6-OHDA rat model of PD (Orosz and Bennett, 1992; Robertson and Robertson, 1989).

After chronic L-Dopa treatment, the efficacy of an acute challenge of L-Dopa to increase DA release is dramatically reduced (**Figure 2**; (Navailles *et al.*, 2011a; Nevalainen *et al.*, 2011). Most importantly, we found that this loss of capacity of 5-HT terminals to release L-Dopa-derived DA is region-dependent. In fact, DA extracellular levels induced by 12 mg/kg of L-Dopa after a 12 mg/kg treatment of L-Dopa for 10 days are reduced by 92%, 79%, 62% in the SNr, HIPP and PFC respectively. In the striatum, DA levels are reduced by 51% after a challenge dose of 12 mg/kg and are not affected after 3 mg/kg (Navailles *et al.*, 2011a). The relatively preserved striatal DA effect after chronic L-Dopa treatment may account for different mechanisms in the striatum compared to other brain structures that could be directly related to the 5-HT terminal heterogeneity within brain regions (**Figure 3**) (Navailles *et al.*, 2011b; Navailles and De Deurwaerdere, 2012b). Notably, the higher striatal DA release could result from a denser striatal 5-HT innervation observed in dyskinetic animals (Gil *et al.*, 2010); but see (Lundblad *et al.*, 2009). Consistently, recent works in rats and marmosets (Rylander *et al.*, 2010b; Zeng *et al.*, 2010) have shown that L-Dopa pharmacotherapy induces a maladaptive plasticity of 5-HT axon terminals in the striatum (increased levels of 5-HT transporter and sprouting of 5-HT varicosities with high synaptic incidence) that may predispose to dyskinesia. Finally, both this aberrant plasticity of striatal 5-HT fibers and the loss of inhibitory tone provided by cortical DA upon subcortical DA function could preserve subcortical DA release after chronic L-Dopa treatment and participate in the emergence of LIDs. Taken together these data indicate that L-Dopa, by mediating its effects via 5-HT neurons, generates different states of DA transmission that evolve in a region-dependent manner over the time-course of the treatment. It can be postulated that the resulted striatal-extrastriatal DA imbalance induced by L-DOPA play a critical role in the development of LIDs (Navailles and De Deurwaerdere, 2012b).

5.2.2.2. Impact of L-DOPA on 5-HT transmission and relationship to LIDs

L-Dopa, by entering 5-HT neurons, mediates numerous changes in 5-HT neuron homeostasis (Navailles *et al.*, 2011a; Navailles and De Deurwaerdere, 2012b). The production of massive amounts of DA has tremendous impact on 5-HT function at the level of the metabolism, the activity and the morphology of 5-HT neurons (Navailles and De Deurwaerdere, 2012b). Some changes in 5-HT indexes have been associated with the emergence of LIDs and should be taken into consideration to better control 5-HT transmission and L-Dopa's side effects (Fox *et al.*, 2009; Navailles and De Deurwaerdere, 2012b; Scholtissen *et al.*, 2006).

Most studies have shown that L-Dopa reduces striatal 5-HT tissue concentrations in chronic L-Dopa-treated rats (Carta *et al.*, 2007; Gil *et al.*, 2010; Gil *et al.*, 2011; Lindgren *et al.*, 2010; Navailles *et al.*, 2011a) although a trend toward an increase was also reported (Carta *et al.*, 2006). These differences may account for the time of sacrifice after the last L-Dopa administration, i.e. 1h, 3h, 24h or more. When considering peak-dose dyskinesias, it is consistently observed that L-Dopa administered within 1 to 3 hours before sacrifice decreases 5-HT tissue levels, while increasing DA tissue levels in the striatum (Carta *et al.*, 2007; Gil *et al.*, 2011; Navailles *et al.*, 2011a). According to these opposite 5-HT/DA changes (Gil *et al.*, 2010), the severity of LIDs is both correlated with striatal DA (positively) and 5-HT tissue levels (negatively and more stringently than DA levels) (Gil *et al.*, 2011). Moreover, an acute treatment with the serotonin precursor 5-hydroxytryptophan 5-HTP, which increases 5-HT and decreases DA tissue levels induced by L-Dopa, is able to reduce the appearance of LIDs (Tronci *et al.*, 2013). Some studies have found a positive correlation between LIDs and 5-HT tissue levels in the striatum (Eskow *et al.*, 2009) and prefrontal cortex (Carta *et al.*, 2006) or no correlation between LIDs and 5-HT tissue levels in the striatum (Carta *et al.*, 2006). In these studies, biochemical measurements were performed within a timeframe that does not allow for a direct correlation with LIDs, i.e. either 72h (Carta *et al., 2006)* or one week (Eskow *et al.*, 2009) after the last L-Dopa administration. Regardless of this timeframe, tissue 5-HT levels in the striatum and cortex, but not the SNr, are systematically found to be higher in dyskinetic versus non-dyskinetic rats (Carta *et al.*, 2007; Gil *et al.*, 2011; Lindgren *et al.*, 2010).

When considering 5-HT extracellular levels and LIDs, two parameters emerge as critical indicators, i.e. the reactivity of 5-HT terminals to L-Dopa challenge (indexed by 5-HT release) and basal 5-HT extracellular levels after chronic L-Dopa treatment. First, the reactivity of 5-HT terminals is modified in a region-dependent manner that echoes the region-dependent ability of L-Dopa to increase DA release after a chronic L-DOPA treatment (see above; (Navailles *et al.*, 2011a). Indeed, the lack of sensitivity of striatal 5-HT terminals to L-Dopa, i.e. no change of 5-HT release after acute or chronic treatment (Lindgren *et al.*, 2010; Navailles *et al.*, 2010b, 2011a; Navailles *et al.*, 2013), is associated with a relatively preserved effect on DA release. On the other hand, the highest sensitivity of 5-HT terminals to L-Dopa observed in the SNr, i.e. potentiation of L-Dopa-induced decrease in 5-HT levels after chronic treatment (Navailles *et al.*, 2011a), lead to the most profound loss of efficacy of L-Dopa to increase DA release (Navailles *et al.,* 2011a). Second and as for 5-HT tissue levels, basal 5-HT extracellular levels are higher in the striatum, but not SNr, of awake dyskinetic compared to non-dyskinetic animals (Lindgren *et al.*, 2010). The authors suggested that the denser 5-HT innervation in the striatum of dyskinetic animals could account for the higher basal 5-HT levels.

Concerning 5-HT terminal density/morphology and LIDs, dyskinetic rats display an increase in AADC protein expression in the lesioned striatum without change in tyrosine hydroxylase expression, an effect associated with a higher 5-HT immunoreactivity and SERT binding densities compared to non-dyskinetic animals (Gil *et al.*, 2010; Navailles and De Deurwaerdere, 2012b; Rylander *et al.*, 2010b). Both SERT binding and tryptophan hydroxylase (TPH) immunolabeling provided evidence that chronic L-Dopa treatment and onset of LIDs are associated with an increased 5-HT innervation and marked hypertrophy of striatal 5-HT axonal varicosities (Rylander *et al.*, 2010b; Zeng *et al.*, 2010). Among species and brain regions examined, these studies reported a sprouting of 5-HT axon terminals in the DA-lesioned striatum and motor-premotor cortices of 6-OHDA rats, in the caudate nucleus and putamen of MPTP-treated monkeys and in the putamen and globus pallidus of parkinsonian patients that develop LIDs (Rylander *et al.*, 2010b; Zeng *et al.*, 2010). Although some authors have found that the increased number of SERT-immunoreactive varicosities is associated with larger amount of stimulated (KCl evoked) [^{3}H]-DA release in striatal slices from L-Dopa-treated dyskinetic rats (Rylander *et al.*, 2010b), others have failed to correlate the higher SERT-positive nerve fiber density in the lesioned striatum of dyskinetic and non-

dyskinetic rats with the magnitude of KCl-evoked DA release measured in vivo by chronoamperometry after chronic L-Dopa treatment (Lundblad *et al.*, 2009). It is noteworthy that the use of SERT binding may not be a faithful index of 5-HT terminal nerve density as regards of the heterogeneous expression of this protein by 5-HT terminals (Amilhon *et al.*, 2010; Navailles and De Deurwaerdere, 2012b). Thus far, we cannot exclude that the 5-HT hyper innervation together with the marked hypertrophy of 5-HT axon varicosities participate in the preserved but erratic release of DA in the striatum throughout L-DOPA treatment.

Among the different 5-HT indexes explored in relation to LIDs, the strongest correlations were found between 5-HT tissue levels or 5-HT terminal density and AIMs scores, in line with the idea that the status of the presynatic DA releasing compartment, namely the integrity of 5-HT neurons, is a critical determinant of both the induction and maintenance of LIDs (Ulusoy *et al.*, 2010). Targeting the 5-HT system has proven to be effective in reducing LIDs though not optimally. Indeed, the antidyskinetic strategies used (i.e., $5\text{-HT}_{1A/1B}$ agonists or 5-HTP) have mostly focused on striatal DA release as an index of efficacy in reducing LIDs (Bezard *et al.*, 2013a; Carta *et al.*, 2007; Lindgren *et al.*, 2010; Rylander *et al.*, 2010b; Tronci *et al.*, 2013). However, the mechanisms by which L-Dopa releases DA from 5-HT terminals are far from being fully elucidated. Different mechanisms (exocytotic versus non-exocytotic) could be triggered regarding the dose of L-Dopa (i.e., high doses favoring the non-exocytotic process) while the region-dependent effects of L-Dopa on 5-HT and DA releases may reflect the regional heterogeneity of 5-HT terminals characterized by the variable expression of numerous regulatory proteins (**Figure 3**) (Navailles *et al.*, 2011b). 5-HT neurons display distinct characteristics at the molecular, i.e. variable expression and sensitivity to $5\text{-HT}_{1A/1B}$ receptors, SERT, VGLUT3, cation channels (Amilhon *et al.*, 2010; Blier *et al.*, 1990; Casanovas *et al.*, 1997; Hervas *et al.*, 1998; Invernizzi *et al.*, 1991; Invernizzi *et al.*, 1997; Kreiss and Lucki, 1994; Romero and Artigas, 1997), anatomical, i.e. from the medial or dorsal raphe nuclei (Azmitia and Segal, 1978; McQuade and Sharp, 1997) and ontogenesis, i.e. pet1-dependent versus pet1-resistant 5-HT neurons (Gaspar *et al.*, 2003; Kiyasova *et al.*, 2011) levels that may participate in the region-dependent changes of 5-HT and DA releases (Navailles *et al.*, 2011b). Beyond this intrinsic 5-HT neuronal heterogeneity, chronic L-DOPA treatment by itself is known to modify the morphology of these 5-HT neurons and the synaptic plasticity in various brain regions (Berthet *et al.*, 2009; Picconi *et al.*, 2010; Picconi *et al.*, 2005; Prescott *et al.*, 2009; Rylander *et al.*, 2010b; Zeng *et al.*, 2010). Therefore, the

relative contribution of the exocytotic and non-exocytotic mechanisms triggered by L-Dopa in the multiple 5-HT-innervated brain regions is currently unknown. This may explain why the stimulation 5-$HT_{1A/1B}$ autoreceptors partially reduce LIDs (Bezard *et al.*, 2013a; Carta *et al.*, 2007; Lindgren *et al.*, 2010) since they only target the exocytotic component of L-Dopa-induced DA release and in a non-optimal manner regarding the cortical/subcortical imbalance in DA transmission induced by L-Dopa.

Finally, LID can occur without enhancement of striatal DA release (Porras *et al.*, 2014). Thus, if the hypothesis of the swings of DA synaptic levels is validated, it might occur in brain areas other than the striatum. As recently suggested, DA swings can be triggered by many mechanisms involved in L-Dopa-stimulated DA release and clearance (Hensler *et al.*, 2013; Navailles *et al.*, 2013).

5.2.3. Impact of L-DOPA on amino acids : relationship to LID

There are many relationships between LID and amino acids. Anatomically, DA and 5-HT receptive cells are GABAergic neurons, interneurons and glutamatergic neurons. In the basal ganglia, it is postulated that the multiple changes occurring in this network and the increased DA function induced by L-Dopa concur to decrease the activity of output GABAergic neurons of the SNr and the GPi (Albin *et al.*, 1989b; DeLong, 1990; Nambu, 2008). Clinically, STN-DBS, the glutamatergic nucleus of basal ganglia, and GPi-DBS efficiently reduce LIDs in humans (Bejjani *et al.*, 2000; Krack *et al.*, 2003; Krack *et al.*, 1999; Krack *et al.*, 1997). In animal models, the data are mitigated regarding the efficacy of STN-DBS on LIDs reporting an exacerbation (Oueslati *et al.*, 2007), a reduction (Simonin *et al.*, 2009) or no alteration (Gubellini *et al.*, 2006).

These differences could account for the fact that the changes of glutamate release induced by L-Dopa depend on the dose, the species and the brain region examined. The acute administration of L-Dopa at 25 mg/kg increases glutamate release measured by intracerebral microdialysis in the striatum of naïve and 6-OHDA rats (Jonkers *et al.*, 2002). After chronic L-Dopa treatment, basal extracellular glutamate levels are consistently increased in the striatum and/or SNr, and associated with an increase in the glial glutamate transporter GLT-1 (Dupre *et al.*, 2011; Robelet *et al.*, 2004). Specifically in dyskinetic animals, basal

extracellular levels of glutamate are similar to those reported in non-dyskinetic rats. However, differences in glutamatergic reactivity are observed on glutamate release induced by various doses of L-Dopa (4, 12 or 100 mg/kg) or a depolarizing stimulus (Dupre *et al.*, 2011; Nevalainen *et al.*, 2013b; Robelet *et al.*, 2004). It has been reported in peak-dose LIDs that glutamate release in the GP and SN was not altered by 15 mg/kg L-Dopa in mice while it was enhanced in the SN, but not the GP or dorsolateral striatum of rats after 6 mg/kg (Bido *et al.*, 2011; Mela *et al.*, 2012). Nevertheless, riluzole, a glutamate release inhibitor alleviates established AIMs in the 6-OHDA-lesioned mouse (Lundblad *et al.*, 2005) and rat (Dekundy *et al.*, 2007). In clinical trials, the effects of riluzole or another glutamate release inhibitor naftazone are mitigated (Bara-Jimenez *et al.*, 2006; Merims *et al.*, 1999; Rascol *et al.*, 2012). Overall, these studies bring evidence for a hyperactivity of glutamatergic neurons in response to chronic L-Dopa that may operate in a region-dependent manner.

Accordingly, L-Dopa is expected to change GABA release in the basal ganglia (Bezard *et al.*, 2001e; Cenci, 2007b). In dyskinetic mice, L-Dopa enhances GABA release in the SNr and GP (Bido *et al.*, 2011). Although this effect is reduced by the non-selective NMDA receptor antagonist amantadine, L-Dopa does not enhance glutamate extracellular levels in both regions (Bido *et al.*, 2011). In dyskinetic rats, peak-dose LIDs are also associated with an increase in GABA release in the SNr (but not in the striatum) that is prevented by amantadine in line with a concomitant surge in nigral glutamate levels (Mela *et al.*, 2012; Mela *et al.*, 2007). These data suggest that the STN may facilitate the activity of the striatonigral GABAergic pathway at terminal level. Indeed, STN-DBS induced forelimb dyskinesia associated with glutamate release in the SNr of hemiparkinsonian rats (Boulet *et al.*, 2006). Interestingly, STN-DBS at a lower intensity that did not induced forelimb dyskinesia, also increased nigral GABA release but without altering glutamate release (Boulet *et al.*, 2006). An enhanced reactivity of GABAergic terminals in the SNr of dyskinetic animals, as also proposed from in vitro studies (Rangel-Barajas *et al.*, 2008) is compatible with a consequent inhibition of the nigrothalamic GABAergic tone (Albin *et al.*, 1989b; Chevalier and Deniau, 1990). Accordingly, L-Dopa decreases GABA release in the thalamus of 6-OHDA rats or MPTP-treated monkeys that displayed severe dyskinesia after 6 months treatment with L-Dopa (Marti *et al.*, 2007; Porras *et al.*, 2014). However, it remains to be determined whether the decrease in thalamic GABA release induced by L-Dopa is magnified in case of peak-dose LID.

In conclusion, the impact of neurostransmitters on peak-dose LID is a complex mechanism regarding the neurochemical environment created by L-Dopa (Navailles and De Deurwaerdere, 2012b). It requires a synaptic increase in DA release, even modest, from 5-HT terminals. High magnitude of variations in synaptic DA levels could favor the development of LID. Such swings and/or aberrant release of DA from L-Dopa may occur in many brain regions other than the striatum. This is an important point because LID is associated with changes of cellular activity in sensorimotor, associative and limbic territories (Bastide *et al.*, 2014; Guigoni *et al.*, 2005c). L-Dopa also alters 5-HT extracellular levels directly and in a region-dependent manner as well as amino acids transmission. Notably, LID is associated with a higher GABA release in the SNr and a lower GABA release in the thalamus. Regarding the presynaptic release of other neurotransmitters, some preclinical studies indirectly suggest that peak-dose LID could also be associated with putative modifications of release in acetylcholine (Di Chiara *et al.*, 1994; Zhang *et al.*, 2013), noradrenalin (Delaville *et al.*, 2011), opioidergic peptides or endogenous cannabinoid reactivity (Huot *et al.*, 2013). Such predictions are expected to be validated soon since the behavioral evaluation of LIDs in animals has been considerably improved in the past years and the *in vivo* assessment of neurochemical environment has benefited from the coupling of intracerebral microdialysis with powerful neurochemical method analysis (Cenci and Ohlin, 2009; Navailles *et al.*, 2013).

5.3. Imaging

5.3.1. Studies of the DA system

Neuroimaging studies have provided *in vivo* support for the importance of pulsatile stimulation of DA receptors in the emergence of LID. DArgic function can be assessed using positron emission tomography with ligands that bind to the vesicular monoamine transporter type 2 (VMAT2), the plasmalemmal DA transporter (DAT) (Au *et al.*, 2005; Brooks *et al.*, 2003) and post-synaptic DA D1 and D2 receptors. Additionally, the fluorinated analog of levodopa, 6-[^{18}F]fluoro-L-Dopa (6FD) can be used to assess uptake and decarboxylation of levodopa to DA, as well as storage of DA in synaptic vesicles and, when prolonged scans (4 hours, rather than the usual 90-120 minutes) are performed, DA turnover (Sossi *et al.*, 2001).

Dyskinesias tend to occur in more advanced PD. One might therefore anticipate a loose relationship between markers of presynaptic DArgic integrity and LID. With the possible

exception of dyskinesias that emerge following fetal mesencephalic transplantation (see below), there is little evidence for this in the literature, apart from a report by Linazasoro and colleagues, who found an inverse relationship between 6FD uptake and dyskinesias (Linazasoro *et al.*, 2004). Fluctuations in motor function, which commonly occur together with dyskinesias, are associated with reduced 6FD uptake (de la Fuente-Fernandez *et al.*, 2000), but there is substantial overlap between patients with and without motor fluctuations, suggesting that other factors play an important role.

Traditional measures of presynaptic DArgic integrity give only a rough estimate of striatal DA nerve terminal density. As discussed elsewhere in this review, a critical factor in the emergence of motor complications is the pattern of DA receptor stimulation. Thus, assessment of the central pharmacokinetics of levodopa action may provide greater insight. As previously described in this review, [^{11}C]Raclopride labels D2/D3 receptors with relatively low affinity and its binding is subject to competition from endogenous DA (Breier *et al.*, 1997; Seeman *et al.*, 1989). Thus, interventions such as levodopa therapy that result in increased synaptic DA will result in reduced [^{11}C]raclopride binding as assessed by PET (Tedroff *et al.*, 1996). De la Fuente-Fernandez et al. found a greater magnitude but less sustained decline in [^{11}C]raclopride binding in PD patients who had a stable response to levodopa at the time of the PET study but who went on to develop motor fluctuations within 3 years compared to those subjects who had stable response to medication 3 years later (de la Fuente-Fernandez *et al.*, 2001b). In a follow-up study, these authors found that the relative change in [^{11}C]raclopride binding one hour after oral levodopa increases with disease duration and even after correction for this factor, is higher in subjects with LID compared to those with a stable response, while there is no difference between dyskinetic and non-dyskinetic subjects 4 hours after levodopa (de la Fuente-Fernandez *et al.*, 2004b). This is compatible with a more pulsatile pattern of levodopa-induced DA release in subjects with motor complications. Similar findings have been reported by Pavese et al. (Pavese *et al.*, 2006).

These findings are reminiscent of the sensitization of DA release that is associated with drug addiction as previously discussed in the review. In contrast to the motor complications, which are associated with sensitized DA release in the putamen (i.e. motor striatum), PD patients with DDS have sensitized DA release restricted to the ventral striatum as assessed by change in [^{11}C]raclopride binding in response to levodopa (Evans *et al.*, 2006). As is the case for ventral striatal release of DA induced by amphetamine in healthy control subjects (Leyton *et*

al., 2002), the change in [^{11}C]raclopride binding correlated with 'drug-wanting' rather than the subjective pleasure or 'liking' of drug.

Another way of looking at the kinetics of DA release and metabolism is to estimate DA turnover using prolonged scans with 6FD. While uptake measured over the standard 90-120 minute scan reflects uptake, decarboxylation to fluoroDA and trapping of fluoroDA in synaptic vesicles, prolonged scans also reflect the egress and subsequent metabolism of this trapped radioactivity. The model used to analyze the acquired radioactivity data thus shifts from one that assumes unidirectional transport of tracer (i.e. the radioactivity is trapped) to a reversible model. The effective distribution volume that is derived from this reversible tracer model correlates well with the inverse of the ratio of tracer loss to tracer uptake constants (Sossi *et al.*, 2001), which in turn correlates with classical neurochemical measures of DA turnover (Doudet *et al.*, 1998). DA turnover measured using this approach is increased early in PD (Sossi *et al.*, 2002) and further increases occur with disease progression (Sossi *et al.*, 2004). Furthermore, even when one accounts for disease severity, the magnitude of the abnormality in DA turnover is greater in PD patients with younger disease onset than the abnormality of 6FD uptake (Sossi *et al.*, 2006). This suggests that comparable degrees of denervation result in greater increases in DA turnover in younger individuals and is in keeping with the widely held view that such individuals are more prone to dyskinesias (Golbe, 1991; Grandas *et al.*, 1999; Quinn *et al.*, 1987) (Kumar *et al.*, 2005).

The determinants of DA turnover are not fully understood. However, it appears that in patients with PD, downregulation of the DAT results in increased turnover, again even after correcting for disease severity (Sossi *et al.*, 2007). One would therefore predict that downregulation of DAT beyond the degree expected based on disease severity (i.e. loss of DA nerve terminals) would be an independent predictor of the development of LID and this indeed appears to be the case (Troiano *et al.*, 2006). Thus, while downregulation of the DAT may serve a useful function in early disease in order to conserve levels of DA in the synapse (Calne and Zigmond, 1991; Lee *et al.*, 2000b), in the long run such a compensatory mechanism may prove deleterious.

Dyskinesias that occur following fetal mesencephalic transplantation may represent a special example, as they may occur either as an exaggerated form of LID or in some patients, may occur off medication (Freed *et al.*, 2001; Olanow *et al.*, 2003). Ma and colleagues reported

post-operative increases in 6FD uptake in the left posterodorsal putamen and left ventral striatum of patients who developed post-transplant dyskinesias (Ma *et al.*, 2002). In contrast, using a combination of 6FD and [^{11}C]raclopride, Piccini et al. found no evidence for increased graft-derived DA release in subjects with dyskinesias (Piccini *et al.*, 2005).

5.3.2. Studies of non-DArgic mechanisms

As reviewed elsewhere in this manuscript, there is extensive evidence from animal models of alterations downstream to striatal DA receptors following chronic DArgic stimulation, thought to contribute to LID. These include upregulation of immediate early genes and of several neuropeptides, including enkephalin and dynorphin. There is very limited evidence available in the imaging literature, largely reflecting the paucity of tools. Piccini and colleagues demonstrated reduced striatal binding of the opioid ligand [^{11}C]diprenorphine in PD patients with LID, presumably reflecting occupancy of striatal opioid receptors due to increased opioid levels (Piccini *et al.*, 1997). Whone and colleagues demonstrated in a preliminary study a reduction in thalamic NK1 neurokinin receptor binding in PD patients with LID (Whone *et al.*, 2002). Whether this represents a loss of NK1 receptors or increased receptor occupancy reflecting increased availability of endogenous substance P is unclear.

Studies of cerebral blood flow can be used to infer changes in patterns of neuronal activity within the basal ganglia and its connections. Hershey et al. used PET with [^{15}]H_2O to study the hemodynamic responses to levodopa in PD patients with and without LID. They found increased cerebral blood flow following levodopa administration in the thalamus of dyskinetic patients, associated with reduced blood flow in primary motor cortex (Hershey *et al.*, 1998). As regional cerebral blood flow predominantly reflects synaptic activity, this finding is compatible with a sensitized response to levodopa in the internal segment of the globus pallidus and while it is not easily explained by standard “box and arrow” models of the basal ganglia (Albin *et al.*, 1989b), it is very much in keeping with the reduction in LID that is consistently reported following pallidotomy (Fine *et al.*, 2000). Sanchez-Pernaute and colleagues have studied the hemodynamic response to a selective DA D3 receptor agonist using fMRI and found that the response was increased in rodent and non-human primate animals with LID (Sanchez-Pernaute *et al.*, 2007), in keeping with in vitro and behavioural evidence (Bezard *et al.*, 2003; Bordet *et al.*, 1997; van Kampen and Stoessl, 2003).

5.3.3. Potential future applications

With the few exceptions noted above, most studies performed to date have focused either on DArgic mechanisms or on patterns of cerebral activation in response to medication. Within the DA system, study of the D3 receptor may be of particular interest, but investigation has been hampered by the lack of selective positron-emitting tracers. Other neurotransmitters of interest with respect to their role in LID include 5-hydroxytryptamine, adenosine, excitatory amino acids, and GABA, but there are no relevant studies, in part reflecting the paucity of informative radioligands. Studies of cell signaling pathways and of immediate early gene expression similarly await the development of better tools for in vivo imaging.

5.4. Electrophysiology

5.4.1. Extracellular

Studies of neuronal activity of the basal ganglia in LID, both in animal models of parkinsonism and in PD patients, have been conducted using mainly two different approaches, single cell and local field potential recordings. In this review, human and non-human primate studies will be summarized.

5.4.1.1. Single cell recording

This kind of recording is obtained using microelectrodes and it provides information about the frequency and the pattern of discharge of single neurons. The classical model of basal ganglia function considered that dyskinesias result from decreased neuronal firing rates in the GPi (Albin *et al.*, 1989b; DeLong, 1990) leading to increased activity of thalamocortical motor circuits. Accordingly, microrecording of the neuronal activity in MPTP-treated monkey with DArgic-related dyskinesias showed a reduction of the firing frequency in the GPi in comparison with the Off state (Boraud *et al.*, 2001; Filion *et al.*, 1991; Heimer *et al.*, 2006; Papa *et al.*, 1999) and with the on state without dyskninesias (Boraud *et al.*, 2001; Heimer *et al.*, 2006; Papa *et al.*, 1999). There was also a change in the firing pattern concomitant with the onset of dyskinesias (Boraud *et al.*, 2001; Heimer *et al.*, 2006). A reduction in the fraction of oscillatory cells and in the oscillatory correlations among neurons was observed in the GPi during the on state, which was higher when LID were present (Heimer *et al.*, 2006).

Although firing rate in GPe neurons was increased in the on state respect to the off state (Boraud *et al.*, 2001; Heimer *et al.*, 2006) no differences either in the firing frequency or pattern of neuronal discharge were encountered between the dyskinetic and non dyskinetic states (Boraud *et al.*, 2001). In contrast, Heimer et al, 2006 observed a reduction in the fraction of oscillatory cells only when LID were present (Heimer *et al.*, 2006). In addition, they observed that the ratio of the mean firing rate of the GPe/GPi increased during the LID recording respect to the on state with optimal recovery of parkinsonism without dyskinesias (Heimer *et al.*, 2006).

Thus, single cell studies in the MPTP monkey indicated that LID would take place when frequency is excessively decreased in the GPi and the firing pattern suffers a change in the oscillatory activity with a reduction in the fraction of oscillatory cells.

In human studies, dyskinesias induced intra-operatively by administration of apomorphine to patients with PD was associated with a reduction of the GPi firing rate respect to the off state (Lee *et al.*, 2007; Levy *et al.*, 2001; Merello *et al.*, 1999a), while differences between on state with and without dyskinesias were not clearly observed (Lee *et al.*, 2007; Levy *et al.*, 2001). The firing pattern was also altered during on state with dyskinesias respect to the off state with an increment in the burst-like (Levy *et al.*, 2001; Merello *et al.*, 1999a) or irregular (Lee *et al.*, 2007) discharges. In contrast, the mean firing rate of the neurons of the STN was not reduced during the on state without dyskinesias respect to the off state while it was significantly reduced when LID were present (Levy *et al.*, 2001). LID were also associated with an increment in the proportion of spikes in burst in the STN, which was not observed during the on state without dyskinesias (Levy *et al.*, 2001). Of note, there was a high variability in the effect of apomorphine upon the firing rate of single STN and GPi neurons (Levy *et al.*, 2001).
Regarding the GPe, just a few neurons have been studied, which had an increment in the firing rate by 50-90% (Lozano *et al.*, 2000).

Thus, in PD patients, LID are associated with reduced firing rate and change in the pattern of GPi neuronal discharges respect to the parkinsonian state, while the major difference between the states on with and without dyskinesias seems to be the firing pattern. In the STN, a reduction in the firing rate marked the presence of LID along with a more bursty or irregular

firing pattern. Findings in the GPe are less consistent and further studies are needed to elucidate its role in LID.

5.4.1.2. Local fied potentials

The implantation of electrodes for DBS in the STN and GPi of patients with PD has allowed recording local field potentials (LFP) and recognizing specific patterns of activity according with the motor states. Thus, information about oscillatory neuronal activity of neuronal populations in the basal ganglia of PD patients with dyskinesias is available more recently.

In the STN, peak-dose LID is associated with an increment in the power of the theta-alpha (4-10 Hz) band with a mean frequency at 8.38 Hz (Alonso-Frech *et al.*, 2006; Foffani *et al.*, 2005). The specificity of this relationship is confirmed by different facts. Firstly, the increment of the power in the theta-alpha bad is only recorded when patients are exhibiting dyskinesias and not during the on-periods without such abnormal movements (Alonso-Frech *et al.*, 2006). Secondly, in patients with unilateral dyskinesias, this oscillatory activity was only recorded in the STN contralateral to the hemi-body where LID was present. In patients in whom LID starts in one hemi-body and then spread to the other side, a gain of power of the theta-alpha activity is firstly recorded in the STN contralateral to the hemi-body where LID starts and then, time-locked with the beginning of LID, in the other STN (Alonso-Frech *et al.*, 2006). In addition, patients who suffered diphasic dyskinesias, which are a subtype of LID that appear typically at the onset and end of levodopa antiparkinsonian action, also exhibit a similar theta-alpha activity (mean frequency 7.38 Hz) during this involuntary movement (Alegre *et al.*, 2012).
The theta-alpha activity associated with peak-dose dyskinesias was mainly generated in the dorsal contacts of the electrode and had clear dorsal distribution in the STN, therefore corresponding to the motor region of the STN (Rodriguez-Oroz *et al.*, 2011).

In the GPi, a study conducted in two patients found a negative correlation between LFP power in the band comprised between 8 and 40 Hz and EMG recording in the contralateral limb with LID (Silberstein *et al.*, 2005). In contrast to the studies in the STN, this correlation was peaking in the 8-12 Hz in one case and the 21-30 Hz in the other case, but in both of the two cases there was a strong negative correlation in the beta band. Beta oscillatory activity is

typically observed in the parkinsonian state and correlates with rigidity and bradykinesia (Little *et al.*, 2012; Lopez-Azcarate *et al.*, 2010). The lowering of the power in this band during LID could be interpreted as an over-reduction of these motor signs during the abnormal excessive movements. For instance, a clinical observation is that rigidity is usually abolished in dyskinetic limbs. In addition, the suppression of the "antikinetic" activity of the beta band could also lead to the release of unwanted motor programs. On the other hand, the discrepancy between GPi and STN activity associated with LID could be due in part to methodological aspects (i.e GPi recording have been conducted in two patients, EMG recorded only in one muscle). On this regard, in one patient with unilateral LID induced by a lesion-like effect of the electrode implanted for DBS in the STN, enhanced STN-GPi coherence at low frequencies (10 Hz) was recorded in the nuclei contralateral to the dyskinetic hemi-body suggesting that an oscillatory activity in the theta-alpha band is probably present along the basal ganglia circuit during LID (Foffani *et al.*, 2005). Although there is no similar record in PD patients with LID, this might also be a feature of this state.

In summary, the classical model of the basal ganglia explained LID as the consequence of an striatal DArgic overstimulation that eventually causes an inhibition of the GPi. However, the fact that lesions of the GPi not only improved parkinsonism but abolished or greatly ameliorated LID proved this concept to be wrong. Interestingly, although in MPTP studies the firing rate of the GPi was lower during the on state with that without dyskinesias, this has not been demonstrated in PD patients as the neuronal firing rate was similar when patients exhibited LID and when they had the antiparkinonian benefit of apomorphine without dyskinesias. In contrast, a more irregular and bursty pattern of discharge has been encountered in all single cells studies undertaken in primates and in humans. The importance of this finding has been somehow reinforced with the LFP recording given consistency to the notions that it was the pattern and not the frequency of discharge the most relevant feature in the pathophysiology of LID. Current interpretation of the benefit of surgical interventions in the GPi (lesion and DBS) are more aligned with a disruption of a DArgic induced abnormal synchronization of neuronal activity along nuclei of the motor circuit.

5.4.2. *Ex-Vivo*

The first paper addressing the electrophysiological plastic changes in neurons recorded from rats displaying dyskinetic movements dates back to 2003 (Picconi *et al.*, 2003). This paper demonstrated for the first time that dyskinetic motor abnormalities are coupled to an absence of bidirectional synaptic plasticity in the striatal projecting neurons (Picconi *et al.*, 2003), opening the way to future studies on the possible synaptic mechanisms underlying LID.

The experimental model chosen is the well characterized unilaterally 6-OHDA-lesioned rat in (Schwarting and Huston, 1996a). Six weeks after the DA denervation, animals lose corticostriatal plasticity, both long term potentiation (LTP) and long term depression (LTD) (Calabresi *et al.*, 1992; Centonze *et al.*, 1999b; Picconi *et al.*, 2003). Notably, the degree of DA denervation influences these two forms of plasticity in different ways, nearly full DA loss blocks the induction of both LTP and LTD, while partial DA depletion allows LTP induction but selectively alters its maintenance, leaving LTD induction and maintenance unaffected (Paille *et al.*, 2010).

Chronic L-Dopa treatment (Cenci *et al.*, 1998; Picconi *et al.*, 2003) at a therapeutic dosage allows to restore LTP in all the parkinsonian rats and to distinguish two different drug-induced behavioral responses. Animals that do not develop dyskinesia, the "therapy responsive" rats, display the anti-parkinsonian effects of the drug and a physiological bidirectional plasticity (LTD, LTP and depotentiation). Conversely, dyskinetic rats show severe LID and a normal LTP while they do not express either LTD or depotentiation induced by a low frequency stimulation (LFS). Notably, the intrinsic properties of striatal medium spiny neurons (MSNs) recorded from dyskinetic and non-dyskinetic rats did not show differences (Picconi *et al.*, 2003). Such a loss of bidirectional plasticity at corticostriatal synapses may cause a pathological storage of nonessential motor information that would normally be erased, leading to the development and/or the expression of abnormal motor patterns. The biochemical studies indicated that the loss of depotentiation observed in dyskinetic animals is attributable to specific changes occurring along the D1 DA receptor signaling pathway leading to abnormally high levels of Thr34-phosphorylated dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) and consequent inhibition of protein phosphatase 1 activity (Picconi *et al.*, 2003; Santini *et al.*, 2010a; Santini *et al.*, 2007). This paper has provided the first demonstration that combining electrophysiological, behavioral

and molecular analysis is possible to study the biological features of LID in rodent experimental models.

A further step forward was the electrophysiological characterization of depotentiation loss in chronically treated PD rats with two different regimen doses of L-Dopa (Picconi *et al.*, 2004); a direct correlation between the daily dosage of L-Dopa and the induction of dyskinetic movements was demonstrated. Moreover, this study establishes a critical pathophysiological link between the lack of synaptic depotentiation and LID expression.

Prolonged L-Dopa treatment remarkably reduces synaptic D1/NMDA receptor complexes in dyskinetic animals without changing their interaction (Fiorentini *et al.*, 2006). However, further complex molecular alterations take place at glutamatergic synapses, and in particular in NMDA GluN2A/N2B ratio subunits composition that are strictly correlated to abnormal synaptic plasticity and motor behavior in dyskinetic condition (Gardoni *et al.*, 2006). Treatment of non-dyskinetic rats with a synthetic peptide (TAT2B) able to affect GluN2B synaptic localization induces a shift toward a dyskinetic motor behavior in the treated rats. This work indicates altered GluN2A/N2B ratio and redistribution of the two subunits between synaptic and extrasynaptic membranes as two important conditions involved in LID induction (Gardoni *et al.*, 2006).
NMDA receptor complex alteration is accompanied by increased striatal levels of αCa2+-calmodulin-dependent protein kinase II (αCaMKII) autophosphorylation, along with a higher recruitment of activated αCaMKII to the regulatory NMDA receptor GluN2A-N2B subunits. The pharmacological normalization of autophosphorylated αCaMKII is able to reverse both the alterations in corticostriatal synaptic plasticity and the motor deficits in PD rats. The same beneficial effects are produced by a therapeutic regimen of L-Dopa (Picconi *et al.*, 2004).
These data support the concept that molecular disturbances of the glutamatergic synapse, initially caused by DA denervation, create a pathological substrate that induces and maintains the overworking synapse at an altered steady-state that might trigger the development of LID (Gardoni *et al.*, 2006; Picconi *et al.*, 2003).

A further advance in the electrophysiological characterization of the plastic changes in dyskinetic condition has been made in a recent study conducted by our group. Starting from the observation that striatal cyclic guanosine monophosphate (cGMP) signalling is decreased

in dyskinetic rats (Giorgi *et al.*, 2008), we explored the possibility that LTD, which strictly relies on the nitric oxide-dependent activation of protein kinase G (PKG), is also altered in dyskinetic rats. Chronic L-Dopa-treated rats developing LID do not show this form of synaptic plasticity (Picconi *et al.*, 2011). Phosphodiesterase (PDE) inhibitors increase cGMP levels leading to the activation of PKG that represents a critical factor for LTD induction following high frequency stimulation (Calabresi *et al.*, 1999; Calabresi *et al.*, 2007; Centonze *et al.*, 1999a). Nitric oxide produced by NOs-positive striatal interneurons activates cGMP/PKG pathway that can be in turns modulated by PDE inhibitors, such as zaprinast and UK-343664 (Calabresi *et al.*, 2007; West and Tseng, 2011). Accordingly, a low dose of PDEs inhibitors applied in vitro rescues *ex vivo* the activity-dependent LTD in striatal slices obtained by dyskinetic rats. Moreover, also intra striatal injection of this drugs in behaving dyskinetic rats rescues LTD, as measured in *ex vivo* slices, and reduces LID (Picconi *et al.*, 2011).

More recently, Usiello and co-workers investigated the contribution of a basal hyperglutamatergic tone in the development of LID and the effect on DA-dependent bidirectional synaptic plasticity (Errico *et al.*, 2011).
Mutant $Ddo^{-/-}$ mice lacking the D-Aspartate Oxidase (Ddo) enzyme, displaying abnormally high levels of the excitatory free D-aspartate and NMDA (Errico *et al.*, 2008), show an aberrant striatal synaptic plasticity. In the MSNs recorded from $Ddo^{-/-}$ mice, similar to what observed in dyskinetic animals, LFS protocol failed to depotentiate the HFS-induced LTP (Errico *et al.*, 2011). When subjected to 6-OHDA lesion, $Ddo^{-/-}$ mice display increased sensitivity to L-Dopa and earlier onset of LID (Errico *et al.*, 2011), further supporting the concept that increased glutamatergic release is a critical risk factor to develop LID.

An interesting work from Grace's group (Belujon *et al.*, 2010) provides evidence in support to a more complex pattern of plastic changes occurring in the striatal output neurons, by studying corticostriatal synaptic plasticity alterations in denervated rats chronically treated with L-Dopa. In particular, the authors studied corticostriatal LTD using *in vivo* extracellular recordings from striatonigral pathway and striatopallidal pathway neurons in anesthetized rats (Belujon *et al.*, 2010). The authors confirm by the use of a *in vivo* experimental approach that LID might be due to an induction of aberrant plasticity; they suppose that this alteration occurs in striatal indirect pathway neurons (i.e. projecting to GPe) combined with an inability

to de-depress established plastic responses in direct pathway neurons (i.e. projecting to GPi/SNr).

A further electrophysiological characterization of the neuronal mechanisms underlying LID come from a study by Gubellini's group (Bennouar *et al.*, 2013) aimed at studying an additional possible therapeutic target, the metabotropic glutamate receptors classes (Cenci, 2007b; Duty, 2012). The metabotropic mGlu4 receptors, are considered a key strategic target for non-DArgic pharmacological treatments of PD and LID. By *in vitro* electrophysiological recordings in corticostriatal slices from 6-OHDA dyskinetic rats the authors have shown the therapeutic effect of a novel and selective mGlu4 receptor positive modulator compound. This drug inhibits corticostriatal synaptic transmission and reduces akinesia when administered in combination with sub-threshold doses of L-Dopa and, notably, also decreases the incidence of LID but not its severity.

Another powerful clinical option alternative to L-Dopa is represented by D2-like receptor agonists, especially in the early stages of the disease, being associated to a reduced risk of dyskinesia development. In advanced stages of PD, D2-like receptor agonists might delay LID appearance and extent. Despite the great attention paid to this DA receptors family, the molecular mechanisms underlying the reduced risk of dyskinesia have not yet been fully characterized.

Finally, recently, Bagetta and colleagues (Bagetta *et al.*, 2012) show that the striatal NMDA/AMPA receptor ratio and the AMPA receptor subunit composition are altered in parkinsonian rats. Interestingly, while L-Dopa treatment fails to restore these synaptic alterations, chronic treatment with pramipexole is associated not only with a reduced risk of dyskinesia but is also able to rebalance, in a dose-dependent fashion, these physiological synaptic parameters, thus providing new insights into the mechanisms of dyskinesia.

5.5. Priming leads to LID

The phenomenon of "priming" has often been called into question to explain the onset of dyskinesia in PD patients on DA replacement therapy. Priming can be defined as the presence of neurochemical and functional aberrant modifications in the DA-denervated basal ganglia

that eventually lead to the emergence of dyskinesia in response to the repeated administration of L-Dopa or DA agonists (Jenner, 2008).

The features of priming have been extensively investigated in experimental models of PD, such as the MPTP-treated primate and the 6-OHDA-lesioned rat (Blanchet *et al.*, 2004; Jenner, 2003a; Morelli *et al.*, 1989; Simola, 2007), although it is in the 6-OHDA-lesioned rat that the great majority of information on the behavioral and neurochemical correlates of priming has been obtained. In this model, priming is usually produced by means of a two-step administration of DArgic agonists, which involves an induction phase and an expression phase, the latter being the step where the effects of priming are evident (Morelli *et al.*, 1989). The manifestation of priming is behavioral, and consists of the emergence of a vigorous, sensitized, contralateral rotational behavior stimulated by a dose of a D1 receptor agonist that is otherwise ineffective in unprimed rats (Morelli *et al.*, 1989; Morelli, 1993). Drug-stimulated contralateral rotations in the 6-OHDA-lesioned rat are indicative not only of antiparkinsonian effects, but also of pro-dyskinetic potential (Lane, 2006); therefore, the sensitized rotational behavior featuring priming may be considered as an index of an abnormal drug-induced motor response (Morelli, 1993). In addition, several studies have shown that priming is associated with a series of neurochemical maladaptive modifications in the DA-denervated striatum that are similar to those observed in animal models of experimental dyskinesia elicited by the chronic administration of DA replacement therapy. These include changes in the production of cyclic adenosine monophosphate (cAMP), phosphorylation of DARPP-32, and expression of mRNAs encoding for immediate early genes, dynorphin, and GAD67, which all critically regulate the activity of the striatal output neurons (Barone, 1994; Carta, 2003; Consolo, 1999; Crocker, 1998; Pinna, 1997; van de Witte, 1998). Interestingly, priming is best manifested when D1, but not D2, receptors are selectively stimulated in the expression phase; moreover, priming is a time-dependent phenomenon, which is only fully expressed after a critical time from its induction has elapsed (Morelli *et al.*, 1989). On the basis of the evidence indicating that D1 receptors play a major role in the emergence of dyskinesia (Aubert *et al.*, 2005; Guigoni *et al.*, 2007), and considering that maladaptive changes produced by DA replacement therapy in the DA-denervated basal ganglia may require some time to develop and thus influence movement performance, the features of priming suggest that this phenomenon could mimic the initial events associated with drug-induced dyskinetic movements.

Further accounting for the existence of similarities between priming and initial events that characterize experimental dyskinesia is the finding that both these phenomena are attenuated by the blockade of glutamate receptors, either ionotropic or metabotropic (Hadj Tahar *et al.*, 2004; Morelli, 1990b; Morin, 2013; van de Witte, 2002). In this regard, it is also noteworthy that cortical glutamatergic efferents form synapses onto striatal medium-spiny neurons of the striatonigral and striatopallidal pathways, and that these synapses demonstrate synaptic plasticity, with the occurrence of LTP and LTD (Kreitzer, 2008). Abnormalities in synaptic plasticity in the DA-denervated striatum have been suggested to play a critical role in the genesis of dyskinesia, by favoring a pathologic form of motor learning following the stimulation of DA receptors by DA replacement therapy (Picconi *et al.*, 2003; Pisani, 2005). Recent studies have shown that priming in the 6-OHDA-lesioned rat may be relevant to the aberrant modifications in motor learning thought to occur in PD. Thus, it has been demonstrated that the performance of rotations during priming induction is necessary for the manifestation of the sensitized motor response on priming expression, as this effect was completely abolished when primed rats were prevented from rotating in response to the initial DArgic challenge (Frau, 2013; Simola, 2009). This finding could indicate that the performance of drug-induced movement upon a first pharmacologic stimulation of DA receptors may generate an aberrant motor memory trace in the DA-denervated striatum, and that this trace may eventually favor the emergence of an abnormal motor response following a later DArgic pharmacologic challenge (Simola, 2009).

It has recently been suggested that the DArgic denervation itself is the major effector of the maladaptive neurochemical and functional changes that underlie dyskinesia, and that priming may not be an absolute requirement for their manifestation (Nadjar *et al.*, 2009). This view is supported in the first place by earlier data obtained in 6-OHDA-lesioned rats showing that the neurochemical effects of priming take place only in the DA-denervated striatum, but not in the intact striatum (Consolo, 1999; Morelli, 1990a). Furthermore, it has been observed in the same model that sensitized rotations induced by a D1 receptor agonist may occur even without prior DArgic stimulation, if this effect is evaluated after a sufficient length of time (e.g. 60 days) from the DArgic denervation (Morelli *et al.*, 1989), thus allowing maladaptive striatal changes to take place. More recently, experiments in MPTP-treated primates have demonstrated that the first administration of L-Dopa elicits neurochemical changes in the striatum that are superimposable to those observed after chronic exposure to the drug (Scholz *et al.*, 2008). In line with this, a study in mice with severe DArgic denervation caused by a

null mutation in the Pitx3 transcriptional factor has shown that dyskinetic movements can be observed even after the first exposure to either L-Dopa or a D1 receptor agonist, without the need for a previous priming (Li, 2013). Whether priming is a phenomenon that exists by itself, being associated with maladaptive neurochemical and functional changes, or it merely consists of the speeding up of aberrant changes that are primarily arising from the DArgic denervation has still to be ascertained. Nevertheless, the possibility may exist that priming associated with DA replacement therapy would affect the propensity of the pharmacologic treatment to elicit dyskinetic movements. Thus, drugs with a marked D1 component (e.g. L-Dopa and apomorphine) have been shown to be the most effective in inducing priming in experimental models, and are also those with the higher dyskinetic potential in the clinical setting. Conversely, drugs that chiefly stimulate D2 receptors (e.g. pramipexole and ropinirole) are less effective in inducing priming, and also have a lower dyskinetic potential, as indicated by clinical evidence showing that the treatment with these agents induces dyskinesia milder than that elicited by L-Dopa.

5.6. Pre-synaptic pathophysiology in striatal medium spiny neurons (MSNs)

Whereas alterations in signaling cascade of striatal medium spiny neurons are ultimately responsible for the appearance of the abnormal motor response to L-Dopa, as they affect gene expression, an increasing body of evidence shows that these alterations are secondary to changes in the presynaptic compartment (relatively to the striatal neurons), which are induced by the progressive loss of the DArgic terminals. Indeed, progression of DA neuron degeneration represents the first and most important risk factor for development of dyskinesia. Accordingly, L-Dopa does not usually induce dyskinesia during the first few years of administration in patients, when sufficient spared DA terminals are present; similarly, partial DA lesioned animals are resistant to development of LID, while complete DA lesioned animals can present dyskinesia already at the first L-Dopa administration. Ulusoy and colleagues have confirmed in an elegant study that the state of the nigrostriatal DArgic compartment determines the susceptibility of rats to the induction of LID; in fact, rats in which the DA levels were reduced by about 70% using a short-hairpin RNA-mediated knockdown of the tyrosine hydroxylase enzyme (shTH), without affecting the integrity of pre-synaptic terminals, were refractory to LID development (Ulusoy *et al.*, 2010). Interestingly, L-Dopa failed to induce dyskinesia in shTH-treated rats even when they were previously

rendered dyskinetic by sub-chronic apomorphine treatment; this suggests that the preserved pre-synaptic DArgic terminals provide a buffering system for the exogenously administered L-Dopa, and mediate regulated release of DA and physiological DA receptor stimulation at striatal neurons (Carta and Bezard, 2011; Ulusoy *et al.*, 2010).

The ability to properly handle the exogenous L-Dopa dramatically diminished as the DA neuron degeneration progresses, and fewer and fewer spared DA terminals can mediate L-Dopa conversion and feedback control release of DA. As previously discussed in this review, recent experimental evidence indicate that when most of DArgic neurons have degenerated serotonin neurons come to play a major role in conversion of L-Dopa to DA, and in the appearance of abnormal movements (Carta and Bezard, 2011; Carta *et al.*, 2007; Munoz *et al.*, 2008). In fact, it is known since early studies that serotonin neurons are able to take up exogenously administered L-Dopa, convert it to DA, and store it into synaptic vesicles (Arai *et al.*, 1995; Arai *et al.*, 1994); this is due to the presence of the same enzymatic machinery expressed by DArgic neurons, i.e., the AACD and VMAT enzymes. Serotonin neurons are expected to contribute to DA release also in early stages of disease; such contribution may initially be beneficial due to the presence of the spared DA terminals that can buffer serotonin neuron-derived DA and avoid excessive DA receptor stimulation (Carta and Bezard, 2011). In support of this view, it has recently been shown that a 30% reduction of striatal L-Dopa-derived dopamine release is induced upon removal of serotonin nerve fibers in intact animals (Nevalainen *et al.*, 2013a).

By contrast, in a situation of advanced dopamine denervation, the serotonin neurons become the main site of L-Dopa conversion to dopamine. In fact, removal of serotonin innervation by 5,7-DHT administration reduced L-Dopa-derived extracellular dopamine levels by about 80% in the striatum of complete dopamine-lesioned rats (Tanaka *et al.*, 1999). However, the loss of spared dopamine terminals, which could buffer serotonin neuron-derived dopamine release, triggers the appearance of dyskinesias due to the absence of a feedback control mechanism for dopamine release on serotonin neurons. In fact, dopamine terminals express the D2 auto-receptor and the dopamine transporter, which can regulate the firing rate of dopamine neurons and the reuptake of dopamine from the synaptic cleft, respectively. The absence of a mechanism of fine regulation of synaptic dopamine levels in serotonin neurons makes serotonin neuron-derived dopamine release uncontrolled, contributing to swings in synaptic dopamine levels, and promoting pulsatile stimulation of striatal post-synaptic dopamine

receptors. In agreement with this view, removal of striatal serotonin terminals by a selective toxin is able to completely suppress LID in 6-OHDA-lesioned rats (Carta *et al.*, 2007; Eskow *et al.*, 2009).

Silencing of serotonin neurons firing can also be achieved by pharmacological targeting of serotonin auto-receptors. According to a major role of serotonin neurons in mediating dopamine release and induction of LID, activation of 5-HT1A receptors (which are mostly located on cell bodies) and/or 5-HT1B receptors (located on axon terminals) was shown to produce a dose dependent reduction of LID. In particular, combination of 5-HT1A and 5-HT1B receptor agonists (8-OH-DPAT and CP-94253, respectively) was found to induce a synergistic effect, with suppression of LID at ineffective doses of the individual drugs (Carta *et al.*, 2007; Munoz *et al.*, 2009; Munoz *et al.*, 2008). Reduction of L-Dopa-derived dopamine release was confirmed to account for the anti-dyskinetic effect in a following microdialysis study (Lindgren *et al.*, 2010). Importantly, this striking anti-dyskinetic effect was observed not only in parkinsonian rats, but also in MPTP-treated dyskinetic macaques (Munoz *et al.*, 2008), suggesting a possible clinical application of this approach to treat dyskinesia. Indeed, a mixed 5-HT1A/1B receptor agonist, eltoprazine, which is currently under clinical investigation for treatment of attention deficit hyperactivity disorder (ADHD), was found to produce complete suppression of LID in both dyskinetic rats and macaques (Bezard *et al.*, 2013b). Albeit partial worsening of the therapeutic efficacy of L-Dopa was seen in those animals following eltoprazine administration, this drug is currently under clinical evaluation for treatment of LID in a small group of patients.

It should be noted that 5-HT1 receptors are not only expressed pre-synaptically, as auto-receptors, but are also present post-synaptically in non-serotonergic neurons; activation of these receptors has been shown to reduce striatal glutamate and GABA release and produce anti-dyskinetic effect (Bishop *et al.*, 2009; Dupre *et al.*, 2008; Zhang *et al.*, 2008). However, it is worth pointing out that combination of 8-OH-DPAT and CP-94253, or administration of eltoprazine at doses able to suppress LID, were shown to be ineffective against dyskinesia induced by apomorphine, suggesting that, at least at moderate doses of 5-HT1 receptor agonists, the anti-dyskinetic effect is due to activation of serotonin auto-receptors (Bezard *et al.*, 2013b; Munoz *et al.*, 2009).

A rat PET-imaging study has recently provided further support for the role of serotonin neurons in mediating L-Dopa-derived dopamine release. In this study, 8-OH-DPAT was found to reverse L-Dopa-induced decrease of [^{11}C]-raclopride binding and increase of extracellular dopamine (Nahimi *et al.*, 2012). Moreover, a very recent work demonstrated that enhancement of the serotonergic tone, by administration of the serotonin precursor 5-HTP, resulted in a reduction of LID, likely mediated by an action of the newly synthesized serotonin on the pre-synaptic auto-receptors, as well as by a partial displacement of L-Dopa-derived dopamine storage from the serotonergic vesicles (Tronci *et al.*, 2013). These results further confirmed the involvement of serotonin neurons in the appearance of LID.

Dampening of serotonin neuron release by 5-HT1 receptor agonists did not only reduce LID, but it has also been shown to prevent induction of post-synaptic alterations at striatal neurons, such as altered NMDA receptor subunits distribution (Munoz *et al.*, 2008). Thus, an overwhelming body of experimental evidence in different animal models suggests that dopamine released as false neurotransmitter from serotonin neurons is the primary trigger of post-synaptic alterations at striatal neurons, which have been associated to dyskinesia.

Clinical feasibility of pharmacological silencing of serotonin neurons to treat dyskinesia in patients has been questioned, due to the possible side effects of 5-HT1 receptor agonists on the therapeutic efficacy of L-Dopa and on mood (particularly in patients that are often affect by mood disturbances). In fact, in advanced stage of disease serotonin neurons may not only be responsible for dyskinesia, but also for the residual therapeutic effect of L-Dopa, as they represent the main site of conversion to dopamine; thus, in a situation of advance dopaminergic degeneration, reduction of LID may be unavoidably accompanied by parallel reduction of the therapeutic effect.

An interesting alternative to selective 5-HT1 receptor agonist may be represented by drugs acting on the serotonin transporter; indeed, in a recent study, selective serotonin transporter inhibitors, such as fluoxetine and citalopram, have been shown to reduce dyskinesia with similar efficacy as 5-HT1 receptor agonists, without affecting the therapeutic efficacy of L-Dopa in rats (Bishop *et al.*, 2012; Conti *et al.*, 2014). While SSRIs appear to exert their action by activation of 5-HT1 auto-receptors, as seen with selective 5-HT1 receptor agonists, they may provide the advantage to reduce dyskinesia without reducing synaptic serotonin levels. This would be mostly important as SSRIs are widely used to treat symptoms of depression in

parkinsonian patients. Moreover, inhibition of neurotransmitter reuptake by SERT blockade may also reduce swings in extracellular dopamine levels. It remains to be established why no anti-dyskinetic effect has been reported in patients under SSRIs treatment, despite their extensive use also in dyskinetic subjects; thus, it is possible that the anti-dyskinetic mechanism is triggered at doses of drugs that are higher than the one used to treat depression. Clinical investigations are warranted to clarify this issue.

Whereas clinical feasibility of serotonin neuron targeting remains to be proved, a recent imaging study has provided important evidence that the serotonin system may play similar role in patients as in animal models. In this study, in agreement with a previous post-mortem investigation (Rylander *et al.*, 2010b), PD patients with LID were shown to have relative preservation of serotonergic terminals compared to patients with stable response to L-Dopa, which correlated with the severity of LID. In patients with LID the same L-Dopa dose induced significantly higher striatal synaptic dopamine levels than in non-dyskinetic patients, in agreement with an earlier PET study (de la Fuente-Fernandez *et al.*, 2004b). Most importantly, the 5-HT1A receptor agonist buspirone, orally administered 15 min before L-Dopa, significantly reduced the L-Dopa-evoked rises in striatal synaptic dopamine release and attenuated LID (Politis, 2014).
Whereas previous studies have reported a partial reduction of LID by buspirone administration (Bonifati *et al.*, 1994), this study provides the first direct evidence that such reduction is linked to reduced synaptic dopamine release.

Overall, an overwhelming body of evidence points to serotonin-neuron derived dopamine release as the single most important determinant of the post-synaptic alterations that characterize LID development.

5.7. Post-synaptic pathophysiology in striatal medium spiny neurons

5.7.1. LID is associated with an increase of IEG expression

The immediate-early genes (IEG) are a class of genes rapidly transcribed in response to an external stimulus (McClung *et al.*, 2004; Okuno, 2011). Although there are a lot of genes potentially involved in LID, the IEG encoding the transcription factor FosB has received particular attention. Indeed, FosB is highly expressed in the dorsolateral striatum of dyskinetic

monkeys and rodents and, especially, its alternatively spliced isoform called ΔFosB (Andersson *et al.*, 1999; Bastide *et al.*, 2014; Berton *et al.*, 2009; Cenci and Konradi, 2010; Cenci *et al.*, 1999; Feyder *et al.*, 2011; Fisone and Bezard, 2011; McClung *et al.*, 2004). In rodents, increased ΔFosB expression is restricted to the striatal MSNs of the direct pathway (Andersson *et al.*, 1999; Darmopil *et al.*, 2009) where activation of extracellular signal-regulated protein kinases (ERK) is also occurring (Darmopil *et al.*, 2009; Santini *et al.*, 2009a). Indeed, ERK activation has been involved in the increase in FosB expression produced by dopamino-mimetic drugs such as cocaine (Zhang *et al.*, 2004). As ΔFosB immuno-reactivity is correlated with the severity of LID in rodents (Andersson *et al.*, 1999; Bastide *et al.*, 2014; Pavon *et al.*, 2006), enhanced expression of ΔFosB appear to be causally related to the development of dyskinesia. Thus, striatal injection of a FosB anti-sense oligonucleotide reduces LID (Andersson *et al.*, 1999). A similar effect has been recently observed, in the macaque, following viral overexpression of a dominant negative of ΔFosB (Berton *et al.*, 2009). Conversely, in the rat, viral vector-induced overexpression of ΔFosB exacerbates LID (Cao *et al.*, 2010).

However, the identifications of specific genes regulated by ΔFosB and implicated in LID remain to be clarified. The increase in FosB-like immuno-reactivity associated with dyskinesia is involved in the up-regulation of mRNA coding for the opioid peptide, prodynorphin, which is selectively expressed by the MSNs of the direct pathway (Andersson *et al.*, 1999). However, a precise assessment of the role played by increased opiod transmission in dyskinesia is complicated by contrasting data on the effects of opioid receptor antagonists on LID (Samadi *et al.*, 2006). Further studies will be necessary to fully characterize the significance of this and other FosB-dependent effects for the development and/or expression of LID.

Zif268 (or NGFI-A/Krox24/Egr1), another immediate early gene coding for a transcription factor is involved in LID. L-Dopa administration increases zif268 in the striatum (Bastide *et al.*, 2014) with an enhanced expression of zif268 mRNA in both striatopallidal and striatonigral MSNs (Feyder *et al.*, 2011). Interestingly, repeated administration of L-Dopa to 6-OHDA-lesioned rats normalizes the levels of zif268 mRNA in the neurons of the indirect pathway, but not in those of the direct pathway (Carta *et al.*, 2005). The lack of normalization of zif268 expression in the MSNs of the direct pathway may be due to the persistent

activation of ERK observed in these cells in association with dyskinesia (Gerfen *et al.*, 2008; Lebel *et al.*, 2010; Pavon *et al.*, 2006; Santini *et al.*, 2007; Westin *et al.*, 2007).

Zif268 promotes the expression of the activity-regulated cytoskeletal-associated protein ARC (or arg3.1) (Li *et al.*, 2005a), an immediate early gene involved in synaptic plasticity (Bramham *et al.*, 2008). Interestingly, LID is accompanied by increased ARC expression in the striatum (Bastide *et al.*, 2014; Sgambato-Faure *et al.*, 2005). In the hippocampus, zif268-induced expression of ARC is invovled in the induction of the late phase of LTP (Li *et al.*, 2005a). Therefore, it is possible that the persistent overexpression of zif268 and ARC is involved in the suppression of depotentiation at corticostriatal synapses, observed in association with LID (cf. above) (Picconi *et al.*, 2003).

5.7.2. Dopaminergic Receptors

5.7.2.1. Canonical pathway

The two major families of dopamine receptors, generally referred to as dopamine D1-type and D2-type, are classically defined by their opposite regulation of cAMP synthesis. Dopamine D1 and D5 receptors, which belong to the type-1 group, are coupled to Gαs/Gαolf proteins, which promote adenylyl cyclase activity and cAMP synthesis. Conversely, dopamine D2, D3 and D4 receptors which constitute the type-2 group, are coupled to Gαi/o proteins, which inhibit adenylyl cyclase and thereby reduce intracellular levels of cAMP (Herve *et al.*, 1993; Stoof and Kebabian, 1981; Zhuang *et al.*, 2000).

Considerable attention has been devoted to the participation of cAMP-mediated signaling in the molecular changes produced by L-Dopa and potentially linked to the development and manifestation of dyskinesia. This line of thought is sustained by the observation that, in rodent and in non-human primate models of PD, dopamine depletion is accompanied by the emergence of a strong sensitization at the level of D1 receptors (**Figure 4**). This, in turn, enhances the effects produced by L-Dopa on dopamine signalling, resulting in abnormal activation of the cAMP intracellular cascade (Alcacer *et al.*, 2012; Feyder *et al.*, 2011; Lebel *et al.*, 2010; Santini *et al.*, 2012; Santini *et al.*, 2010a; Santini *et al.*, 2007).

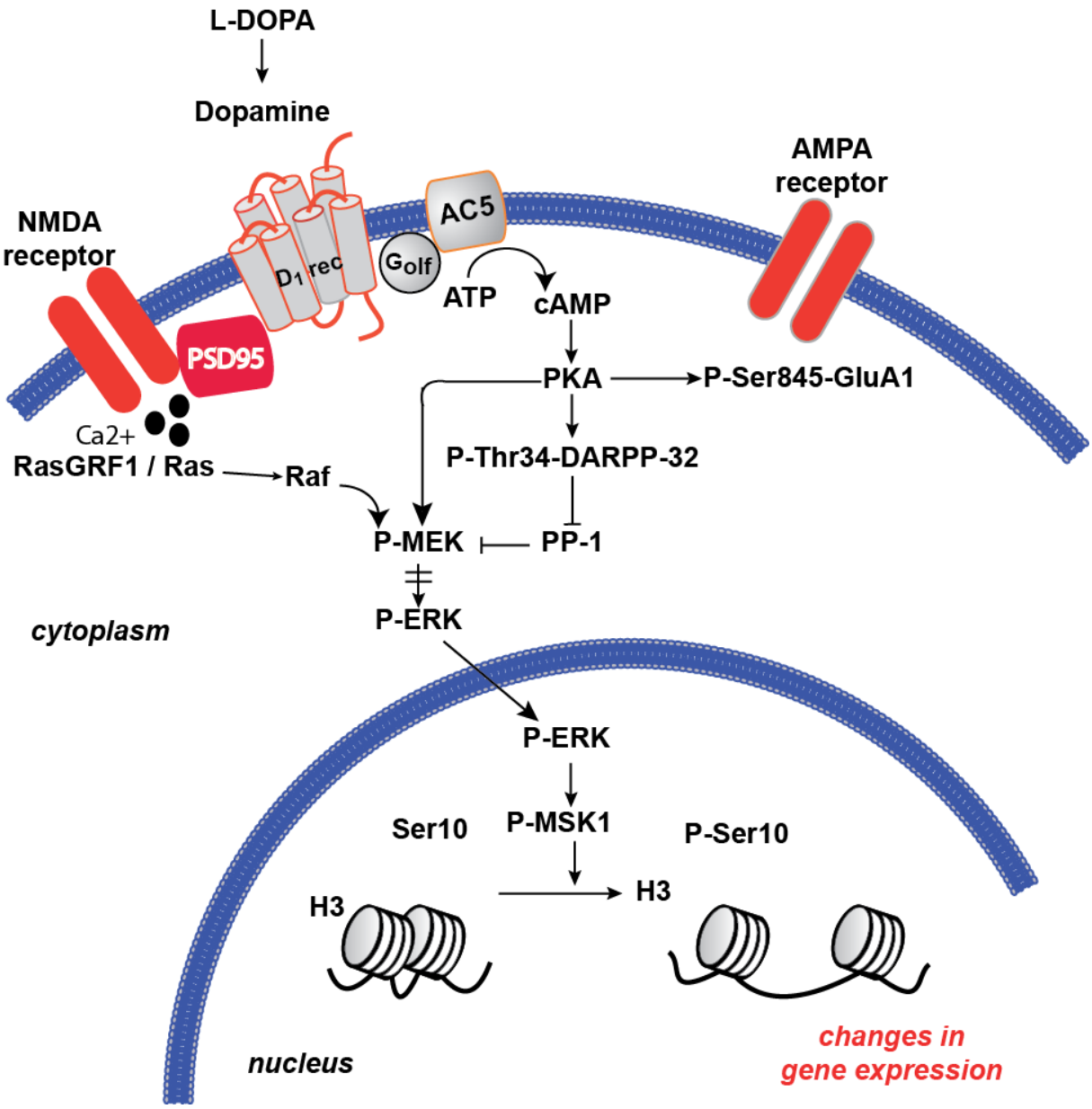

Figure 4. D1 receptor (D1R) signalling. In PD, the loss of striatal dopamine leads to sensitization of D1R on the striatonigral MSNs of the direct pathway. Chronic administration of L-Dopa increases the levels of membrane-bound D1R, thereby exacerbating D1R sensitization and dyskinetic behavior. Sensitized-D1R transmission may be caused by increased levels of adenylyl cyclase 5 (AC 5) in striatonigral MSNs. Increased responsiveness of the D1R/Gαolf/AC5 machinery to L-Dopa results in augmented synthesis of cAMP and hyper-activation of PKA and DARPP-32. Abnormal PKA/DARPP-32 signalling increases the phosphorylation of AMPA GluA1 subunit. This effect promotes the excitability of MSNs and may participate in the loss of corticostriatal LTD and depotentiation associated to LID. Sensitized D1R, cross talking to glutamate signalling (mainly NMDA receptor), lead also to activation of ERK, which controls transcriptional and translational processes. PKA/DARPP-32 and ERK/MSK1 signalling lead to phosphorylation of histone H3 in the nucleus, inducing changes in gene expression.

Studies in experimental models of PD indicate that the number and affinity of D1 receptors is unchanged following dopamine depletion (Aubert *et al.*, 2005; Breese *et al.*, 1987; Joyce, 1991; Marshall *et al.*, 1989; Savasta *et al.*, 1988). Similar results were obtained in post-mortem samples from parkinsonian patients (Hurley *et al.*, 2001; Pimoule *et al.*, 1985; Shinotoh *et al.*, 1993). However, the loss of dopaminergic input to the striatum and the development of dyskinetic behaviour in response to chronic administration of L-Dopa are accompanied by increased recruitment of D1 receptors at the plasma membrane of MSNs, which may be caused by impaired receptor internalization and trafficking (Berthet *et al.*, 2009; Guigoni *et al.*, 2007).

In addition to this phenomenon, other changes have been proposed to contribute to the increase in D1 receptor transmission associated to LID. Studies performed in 6-OHDA-lesioned rats and in post-mortem samples from parkinsonian patients showed that loss of striatal dopamine is accompanied by increased levels of Gαolf (Alcacer *et al.*, 2012; Corvol *et al.*, 2004; Herve *et al.*, 1993; Rangel-Barajas *et al.*, 2011). In 6-OHDA lesioned rats, Gαolf overexpression subsides during chronic L-Dopa administration and does not correlate with the severity of LID (Corvol *et al.*, 2004; Rangel-Barajas *et al.*, 2011). In contrast, in the mouse, elevated Gαolf has been associated with LID (Alcacer *et al.*, 2012). However, in the same animal model, reduced expression of Gαolf did not reduce dyskinetic behaviour (Alcacer *et al.*, 2012) (cf. below).

Another signalling component potentially responsible for the D1 receptor sensitization caused by dopamine depletion and associated to LID is adenylyl cyclase type 5, which is highly expressed in striatal MSNs (Glatt and Snyder, 1993; Mons and Cooper, 1994) and is stimulated in response to D1 receptor-mediated activation of Gαolf (Herve *et al.*, 1993; Zhuang *et al.*, 2000). Evidence obtained using 6-OHDA-lesioned rats shows that dopamine depletion increases the levels of this enzyme in the striatum (Rangel-Barajas *et al.*, 2011). A similar increase is also observed in the substantia nigra pars reticulata, which is innervated by the D1 receptor-expressing striatal MSNs of the direct pathway (cf. above) (Rangel-Barajas *et al.*, 2011). Interestingly, these effects are maintained during repeated administration of L-Dopa, but only in animals displaying severe dyskinesia (Rangel-Barajas *et al.*, 2011).

Taken together the results of the studies described above indicate that the persistent sensitization of D1 receptors associated to LID can be accounted for by increased recruitment of D1 receptors at the cell surface and by overexpression of adenylyl cyclase type 5 in the striatal MSNs of the direct pathway (Berthet *et al.*, 2009; Guigoni *et al.*, 2007; Rangel-Barajas *et al.*, 2011). Altogether, these modifications are likely to influence dopaminergic transmission in the striatum and may underlie the enhancement in the ability of L-Dopa to increase the levels of cAMP and to activate cAMP-dependent protein kinase (PKA). The importance of augmented PKA activity in dyskinesia is indicated by the observation that, in 6-OHDA-lesioned rats, intrastriatal injections of the PKA inhibitor Rp-cAMPS reduces LID (Lebel *et al.*, 2010).

Striatal MSNs express high levels of DARPP-32, which is phosphorylated by PKA on a specific threonyl residue (T34). Phosphorylation at T34 converts DARPP-32 into a selective inhibitor of protein phosphatase-1 (PP-1). This, in turn, suppresses the dephosphorylation of numerous downstream targets of PKA, thereby amplifying behavioral responses produced by activation of cAMP signalling (Borgkvist and Fisone, 2007; Fienberg *et al.*, 1998; Greengard, 2001). Several lines of evidence indicate that PKA-mediated phosphorylation of DARPP-32 is implicated in dyskinesia. Experiments performed in rodents and non-human primates show that LID correlates with increased levels of DARPP-32 phosphorylated at T34 (Lebel *et al.*, 2010; Picconi *et al.*, 2003; Santini *et al.*, 2012; Santini *et al.*, 2010a; Santini *et al.*, 2007). Moreover, this effect is exerted specifically in the striatal MSNs of the direct pathway, which express D1 receptors (Santini *et al.*, 2012). Knock out of DARPP-32, or mutation of the phosphorylation site for PKA (T34), attenuates L-Dopa-induced dyskinesia (Santini *et al.*, 2012; Santini *et al.*, 2007). A similar reduction of dyskinetic behaviour is also observed in mice in which DARPP-32 is selectively inactivated in the striatal MSNs of the direct pathway (Bateup *et al.*, 2010). Interestingly, in MPTP lesioned non-human primates, increased phosphorylation of DARPP-32 persists for up to three months of L-Dopa chronic administration, suggesting that DARPP-32 is implicated not only in the development but also in the maintenance and manifestation of LID (Santini *et al.*, 2010a).

The abnormal activation of PKA/DARPP-32 signalling observed in experimental models of LID may have profound repercussions on synaptic plasticity. As shown by Picconi et al. (2003) LID is associated with blockade of depotentiation at corticostriatal synapses. Notably, depotentiation is prevented by inhibition of PP-1 (Picconi *et al.*, 2003). Therefore, it is

possible that the increase in DARPP-32 phosphorylation associated to LID contributes to the elimination of depotentiation by reducing PP-1 activity. Another possible mechanism by which increased PKA/DARPP-32 signalling prevents depotentiation involves changes in the state of phosphorylation of the GluA1 subunit of the a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor. Increased PKA-dependent phosphorylation of GluA1 at Ser845 correlates with dyskinetic behavior (Santini *et al.*, 2007). This effect is strictly dependent on concomitant phosphorylation of DARPP-32, since it is abolished in DARPP-32 knock out mice (Santini *et al.*, 2007). Phosphorylation of GluA1 at Ser845 promotes glutamatergic transmission (Banke *et al.*, 2000; Mangiavacchi and Wolf, 2004) and may participate in the block of depotentiation observed in dyskinetic rats (Picconi *et al.*, 2003).

In conclusion, sensitized D1 receptor signalling along the canonical cAMP pathway is required for the development and manifestation of LID. The identification of downstream targets of PKA ultimately responsible for the emergence of dyskinetic behavior represents a promising avenue with regard to the development of efficacious anti-dyskinetic therapies. Several questions remain to be addressed. For instance, it has been shown that the reduction of L-Dopa-induced phosphorylation of DARPP-32 and GluA1, achieved through downregulation of Gαolf, does not affect dyskinesia (Alcacer *et al.*, 2012). This finding contrasts with other studies, indicating that inactivation of the PKA/DARPP-32 cascade attenuates dyskinesia (Bateup *et al.*, 2010; Lebel *et al.*, 2010; Santini *et al.*, 2012; Santini *et al.*, 2007) and prompts to a more in-depth analysis of the mechanisms associated to this disorder. In this regard it is particularly important to consider the cross-talk between cAMP signalling and transduction pathways implicated in synaptic plasticity, such as those controlled by the extracellular signal-regulated protein kinases (ERK) and the mammalian target of rapamycin (mTOR).

5.7.2.2. Non-canonical pathways

Newer evidence indicates that D1 receptor do crosstalk to glutamate signaling (mainly NMDA receptors) and thus can engage additional non-canonical pathways in LID (**Figure 4**). The best-characterized pathways implicated in LID are the Ras-ERK and the mTORC1 cascades, which also exert important function in a number of neuronal processes, including learning and memory (Costa-Mattioli *et al.*, 2009; Fasano and Brambilla, 2011). In the now

classical paper by Gerfen et al, ERK dependent signalling was shown to be aberrantly hyperactivated in the dopamine depleted striatum, following D1 receptor activation. Accordingly, this early report also demonstrated that phospho-ERK (pERK) positive signal, a measure of ERK phosphorylation and activation, in the dopamine depleted brain, was almost entirely restricted to enkephalin negative cells, i.e. medium spiny neurons of the direct pathway (dMSNs) (Gerfen *et al.*, 2002b). Hence, these initial observations led to the hypothesis that denervation following 6-OHDA injection could cause a sensitization of D1 receptors, which later was also confirmed by the same authors using the BAC transgenic mice expressing EGFP in either MSNs of the indirect pathway (iMSNs) or dMSNs, upon challenge with amphetamine (Gerfen *et al.*, 2008). Indeed, this selectivity in the ability to activate ERK signalling in a specific subset of MSNs in response to dopaminomimetic drugs is not limited to the DA depleted striatum but can be seen also in the intact brain in response to psychostimulants like cocaine, as originally shown by Caboche and collaborators (Valjent *et al.*, 2000). Importantly, this cellular condition may be at the basis of the motor inducing behavior elicited by both psychostimulants in the normal brain and by dopaminergic agonists (including L-Dopa) in the DA depleted brain since it favors the activity of the direct striatal pathway which following the Albin-Delong model, has a positive effect on the basal ganglia output (Albin *et al.*, 1989b; DeLong, 1990).

Notwithstanding, the first reports clearly implicating an abnormal ERK activation in response to L-Dopa came a few years later. In 2006, Pavon et al reported that pERK can be significantly increased in the denervated striatum with a single administration of L-Dopa and further enhanced with a chronic treatment over 25 days, using high doses (25 mg/kg) (Pavon *et al.*, 2006). Importantly, pERK enhancement in the chronic L-Dopa condition was also found associated to a significant accumulation of FosB/ΔFosB which was previously found accumulating in the striatum both in response to cocaine and in a rat model of LID (Andersson *et al.*, 1999; Kelz *et al.*, 1999).

One important confirmation of the crucial role of ERK signalling in LID came out one year later from a study of the Cenci lab in rats (Westin *et al.*, 2007). In the DA depleted hemiparkinsonian rats, both acute and chronic L-Dopa administration rapidly activate ERK in the medial and lateral striatum, as early as 20 min after, and persists up to 120 min, a time window which parallels the peak of AIMs. Interestingly, at 24h, ERK activation was back to the basal levels. Consistently with a causal engagement of ERK signalling in LID, a clear

correlation was seen between the severity of the AIMs profile and the intensity of ERK activation, which was also confirmed by measuring the phosphorylation of MSK-1, a nuclear protein kinase and direct substrate of ERK proteins. Also, bromocriptine, an antiparkinsonian drug that causes little LID symptoms, did not induce a significant increase in either pERK or pMSK-1, further strengthening the link between ERK and LID. A direct pharmacological confirmation that L-Dopa induces ERK activation specifically in dMSNs was provided by showing that a D1 receptor antagonist, SCH23390, completely suppressed pERK and pMSK-1 induction, as well as FosB/ΔFosB accumulation. On the contrary, raclopride, a D2 receptor antagonist, failed to prevent ERK signalling activation in response to L-Dopa. The direct link between LID, D1 receptor and ERK activity was also later substantiated by the Moratalla group, by showing that genetic ablation of D1 but not D2 receptors suppresses AIMs in the rat and concomitantly prevents ERK phosphorylation, phospho-acetylation of Histone H3 (pAcH3), a direct substrate of MSK-1 and, FosB/ΔFosB accumulation (Darmopil *et al.*, 2009). Finally, upregulation of pERK and pAcH3 levels specifically in the dMSNs was later corroborated using the aforementioned BAC transgenic EGFP expressing mice (Santini *et al.*, 2009a).

These observations did ascribe a pivotal role of ERK signalling in LID. However, they did not demonstrate specifically that a reduction of the activity of this signal transduction pathway could ameliorate the dyskinetic symptoms. The initial evidence was provided in 2007 by the Fisone lab, in mice (Santini *et al.*, 2007). In this paper it was shown that pERK increase well correlated with AIMs severity, as well as the enhancement of phosphorylation of GluA1 (pSer845) and most importantly DARPP-32 (pThr34) (Greengard *et al.*, 1999). Previous work had shown that in striatal cells, active DARPP-32 (pThr34) could stimulate ERK activity by suppressing the activity of protein phosphase STEP, a direct substrate of PP-1 (Valjent *et al.*, 2005). Hence, in the DARPP-32 KO animals, not only AIMs are significantly attenuated but also pERK and pGluA1 are reduced. Finally, systemic administration of SL327, a specific inhibitor of the MEK1/2 kinases upstream of ERK1/2, robustly attenuated LID in mice, providing not only a conclusive demonstration that aberrant ERK activity is part of the pathophysiology of LID but also open interesting therapeutic possibilities for treating dyskinesia by targeting this signaling pathway. Indeed, an initial attempt to translate these findings in a potential therapy was based on the use of the lovastatin, which besides its wide use to treat hyperlipidemia in humans, has also been shown to prevent the membrane localization of Ras proteins, the upstream activators of ERK signalling, effectively reducing

the activity of this cascade in vivo in the brain (Li *et al.*, 2005b). In the rat model of LID, treatment with lovastatin effectively prevented LID formation and reduced both pERK induction and FosB/ΔFosB levels (Schuster *et al.*, 2008). Unfortunately, while the treatment with another statin, simvastatin, did reduce LID and attenuate ERK signaling in the non-human primate model of PD and LID, a pilot trial with a small group of patients failed to reveal any therapeutic effect (Tison *et al.*, 2013), at a dose of 40 mg per day.

Despite this initial negative result, interesting alternative approaches are available to reduce ERK activity in dyskinesia in order to attenuate LID. For instance, indirect manipulation of ERK activity can be achieved either by modulating both group I mGluRs (mGLUR1 and 5) using specific antagonists or Nociceptin/Orphanin FQ receptors using specific agonists (Marti *et al.*, 2012; Rylander *et al.*, 2009). In both cases, pharmacological treatments in experimental rodent models did not only reduce AIMs but also attenuate pERK.

One of the potential problems in LID research is the fact that targeting certain signalling intermediates or receptors aberrantly altered in the initial phases of L-Dopa exposure (priming) may be effective in reducing dyskinetic symptoms but this therapeutic effect may be lost at later stages, a condition more relevant to most PD patients, due to tolerance or additional compensatory cellular mechanisms. Some recent evidence tends to suggest that ERK activity may decline during chronic L-Dopa treatment. On one side, in the non human primate model, levels of phosphorylation of both ERK1/2 and of the ribosomal protein S6 (pS235/236), an indirect cytoplasmic target of ERK, were found maximal upon initial L-Dopa treatment but then declined significantly after 3 months treatment, although did not go back to the basal level (Santini *et al.*, 2010a). This changes were not observed for either DARPP-32 (Thr34) or GluA1 (Ser845), suggesting that while ERK signaling may be more implicated in priming, cAMP signalling may be still relevant for the expression of dyskinesia. However, the situation may not be that simple since in heterozygous mice for Gαolf, cAMP signalling is attenuated while ERK activity remains high. Since these mice do not show significant reductions in AIMs, one can conclude that ERK is more relevant for dyskinesia than the PKA pathway (Alcacer *et al.*, 2012).

Till recently, the investigation of the role of ERK in brain functions in general and in LID in particular has been largely limited to the core components of this signalling pathway, i.e. MEK1/2 and ERK1/2 protein kinases. However, upstream mechanisms connecting both

dopamine D1 and glutamate receptors have been proved to be relevant in the onset of dyskinesia. Ras-GRF1 (**Figure 4**), is a neuronal specific and striatal enriched guanine-nucleotide exchange factor for Ras proteins, previously implicated in cognitive processing as well as in synaptic plasticity and acting as a signalling integrator between D1 receptors and glutamatergic ionotropic receptors (Brambilla *et al.*, 1997; Fasano and Brambilla, 2011; Fasano *et al.*, 2009). In 2010, Fasano et al showed that genetic ablation of Ras-GRF1 in the mouse significantly ameliorate AIMs by reducing both pERK and FosB/ΔFosB levels (Fasano *et al.*, 2010). Importantly, suboptimal doses of SL327, the MEK inhibitor, potentiate the antidyskinetic effect observed in the Ras-GRF1 KO mice, suggesting that a combination therapy targeting both upstream and downstream components of the Ras-ERK pathway may be more effective for treating LID symptoms. The relevance of these observations was also supported by a gene therapy approach in the non human primate model, in which fully dyskinetic monkeys were injected with lentiviral vectors (LV) expressing a cocktail of Ras-GRF1 and ERK dominant negative constructs. This treatment significantly reverted LID symptoms without attenuating the antidyskinetic action of L-Dopa, strongly supporting the idea that Ras-ERK inhibition in already affected individuals may provide a valid therapeutic approach for LID. The fact that Ras-GRF1 inhibition does not completely suppress dyskinetic symptoms may imply that other exchange factors for Ras-proteins could also be implicated in this process. Two valid candidates may be CalDAG-GEFI and CalDAG-GEFII, two striatal enriched Ras-ERK regulators, whose levels were shown altered upon dopamine depletion and L-Dopa treatment (Crittenden *et al.*, 2009). Also, direct coupling of D1 receptor to ERK signalling is believed to play a crucial role in LID. In this respect, recent evidence has elucidated a novel mechanism in which the D1 receptor-mediated ERK1/2 activation in the striatum is dependent on the formation of a signalling complex containing the protein tyrosine phosphatase Shp-2 that persists in dyskinetic animals (Fiorentini *et al.*, 2011; Fiorentini *et al.*, 2013). Thus, Shp-2 may become in the near future an additional interesting target associated to the striatal ERK signalling.

It is well recognized that ERK cascade is a crucial transducer transmitting signals from the cytoplasm to the nucleus. Indeed ERK activity is required in the modulation of protein translation mainly through the phosphorylation of two protein kinases, S6K1 and Mnk1 (Feyder *et al.*, 2011; Santini *et al.*, 2010b). However, another intracellular pathway, partially interacting with ERK, has also been implicated in the translation machinery, the mammalian target of rapamycin (mTor) cascade. This pathway required the formation of the active

complex mTORC1 that contains mTor kinase and is able to phosphorylate and activate S6K1. This complex can be inhibited by rapamycin and also newer drugs with similar mechanisms of action. The first evidence, in mice, of an involvement of mTORC1 in dyskinesia came out in 2009, from the Fisone lab (Santini *et al.*, 2009b). Similarly to ERK, mTor hyperactivation specifically occurs in dMSNs of dopamine-depleted animals challenged with L-Dopa and the degree of phosphorylation of several markers downstream to mTORC1 correlate well to the severity of AIMs. Recently, in the 6-OHDA rat model, it has been confirmed that pretreatment of rapamycin causes a significant reduction and shortening of the dyskinetic profile (Decressac and Bjorklund, 2013), further supporting the idea that excessive de novo protein translation is part of LID pathophysiology. Remarkably, an upstream component of the mTor pathway, Rhes, has proven to be involved in the development of LID, further expanding the list of potential therapeutic targets (Subramaniam *et al.*, 2012).

In recent years, the idea of combination therapy has risen as an interesting concept in optimizing novel therapeutic approaches, with clinical trials targeting both Ras-ERK and mTOR cascades already ongoing in oncology (Chappell *et al.*, 2011). The data available on non-canonical intracellular signalling pathways certainly suggest that a similar path could also be taken to treat dyskinesia.

5.7.3. Glutamatergic receptors

In the last decade, several studies indicated that dysfunctions of the glutamatergic system play a key role in both PD and LID (Calabresi *et al.*, 2010). Alterations in the corticostriatal glutamatergic transmission have been reported in animal models of PD and LID (Mellone and Gardoni, 2013; Sgambato-Faure and Cenci, 2012), as well as in PD patients at different disease stages (Ahmed *et al.*, 2011). Particularly, the subcellular organization and the functional interactions of glutamate receptors in the striatum appears to be critical both in the pathogenesis of PD and in the development of LID.

5.7.3.1. NMDA

After chronic L-Dopa treatment, adaptive changes in the glutamatergic signalling from the cortex to the striatum lead to an aberrant functioning of NMDA receptors at the dendritic spines of striatal MSNs. Since NMDA receptor antagonists have been shown to exert a

beneficial effect in blocking the development of dyskinesia in experimental models of LID (Hadj Tahar *et al.*, 2004; Nash *et al.*, 2004; Wessell *et al.*, 2004), this classical pharmacological approach was brought into clinical trials to reduce the receptor activity in L-Dopa treated dyskinetic patients. Among others, amantadine, a low-affinity, non-competitive antagonist of NMDA receptors (Kornhuber *et al.*, 1991) exhibits anti-dyskinetic activity in PD patients, even though its beneficial effect is attenuated after few months (Sawada *et al.*, 2010; Wolf *et al.*, 2010). However, a recent meta-analysis confirmed at least the short-term benefits of amantadine in the treatment of dyskinesia (Elahi *et al.*, 2012).

Besides NMDA receptor overactivation, alterations in the physiological trafficking and localization of the receptor regulatory subunits at the postsynaptic membrane characterise several neurodegenerative disorders (Mellone and Gardoni, 2013; Sanz-Clemente *et al.*, 2013). Consequently, restoring the physiological synaptic NMDA receptor subunit composition could represent an innovative and relevant therapeutic strategy to be explored in the close future. Interestingly, a great number of studies have addressed the role of synaptic distribution and phosphorylation state of the specific subtypes of NMDA receptors in animal models of LID. Alterations in the localization of NMDA receptor subunits at the striatal synapse have been described in both DA-denervated rats (Picconi *et al.*, 2004) and L-Dopa-treated dyskinetic monkeys (Hallett *et al.*, 2005), even if the mechanisms regulating NMDA receptor subcellular trafficking and function in experimental parkinsonism are far from being elucidated. In particular, chronic L-Dopa treatment results in an abnormal NMDA receptor composition and function at dendritic spines of striatal MSNs. In physiological conditions, GluN2B-containing NMDA receptors are enriched at MSN synapses. However, in L-Dopa-treated dyskinetic rats, GluN2B is redistributed to the extrasynaptic membrane, while the synaptic levels of GluN2A are significantly increased (Gardoni *et al.*, 2006). These events are paralleled by modifications in the association of GluN2B subunit with members of the PSD-MAGUKs family. Moreover, treatment of non-dyskinetic animals with a cell-permeable peptide (CPP) able to reduce the synaptic localization of GluN2B–containing NMDA receptors caused the appearance of dyskinetic behaviours, confirming the importance of a correct balance of NMDA receptor regulatory subunits at synaptic sites (Gardoni *et al.*, 2006). Nash and co-workers (2005) have also highlighted the role of PSD-MAGUKs in these pathological events and suggested that the onset of LID is associated with an increase in PSD-95 and SAP97 at the synaptic membrane. Moreover, treatment with a CPP disrupting GluN2A/PSD-MAGUKs interaction demonstrated that a decrease in synaptic GluN2A-

containing NMDA receptors induces a significant reduction in the onset of LID in 6-OHDA-lesioned rats (Gardoni *et al.*, 2012).

Early work addressing alterations in the phosphorylation state of NMDA receptor subunits identified increased levels of GluN2B-ptyr1472 in different animal models of LID (Oh *et al.*, 1998; Quintana *et al.*, 2010), thus suggesting a reduction of AP-2-mediated endocytosis and the consequent increase in surface GluN2B (Sanz-Clemente *et al.*, 2013). Considering these findings, GluN2B-selective antagonists appeared to be promising for the treatment of LID. Notably, a randomized, double-blind, placebo-controlled clinical trial showed that GluN2B antagonist CP-101,606 was capable to reduce the severity of LID, but induced dose-related dissociation and amnesia (Nutt *et al.*, 2008). However, other recent studies provided contradictory results on the effects of GluN2B-selective antagonists on the onset of LID in experimental models of parkinsonism (Nash *et al.*, 2004; Rylander *et al.*, 2009; Wessell *et al.*, 2004).

Overall, the above-mentioned data further support the idea that molecular disturbances of the NMDA receptor complex in the glutamatergic synapse, initially caused by DA denervation, can create a pathological substrate that may have a causal role in the development of LID.

5.7.3.2. AMPA

AMPA receptors are highly dynamic in terms of phosphorylation and insertion/endocytosis at the postsynaptic membrane (Shepherd and Huganir, 2007). Consequently, understanding the molecular mechanisms, which control the receptor trafficking, is essential to highlight AMPA receptor involvement in neurological disorders.

Alterations of synaptic AMPA receptor expression, subunit composition and phosphorylation have been observed in animal models of LID and in PD patients. An increase in AMPA receptor binding has been reported in the lateral striatum of dyskinetic animals (Calon *et al*., 2002; Ouattara *et al*., 2010b) and in PD patients (Calon *et al*., 2003). Enhanced AMPA receptor subunit phosphorylation and trafficking to striatal synapses have been also described in experimental models of LID (Ba *et al*., 2006; Santini *et al*., 2007; Silverdale *et al*., 2010). In particular, increased PKA-dependent phosphorylation of GluA1-S845, which increases surface expression of AMPA receptors, has been found in rodent models of LID (Ba *et al*.,

2006; Errico *et al*., 2011; Santini *et al*., 2007).

Besides NMDA receptors, modifications of the subunit composition have been also reported for AMPA receptors. A recent study described an alteration in the ratio between synaptic membrane-associated and vesicular GluA2/3 versus GluA1 subunits in the non human primate model of LID (Silverdale *et al*., 2010). Notably, no changes in the total striatal levels of any AMPA receptor subunit has been observed (Hallett *et al*., 2005; Silverdale *et al*., 2010), indicating the redistribution of the receptor subunits, in particular of GluA2/3, from the vesicular fraction to the postsynaptic membrane in dyskinesia (Silverdale *et al*., 2010). Moreover, Ca^{2+}-permeable AMPA receptors and an increase in GluA1 and GluA2 flip isoforms have been involved in both the induction and subsequent expression of LID (Kobylecki *et al*., 2010; Kobylecki *et al*., 2013).

Finally, aberrant function of AMPA receptors also appears to play a key role in the induction of LID. Studies performed in preclinical dyskinesia models indicate that selective AMPA antagonists can be effective in reducing LID (Juranyi *et al.*, 2004; Kobylecki *et al.*, 2010; Konitsiotis *et al.*, 2000), thus confirming a role for overactive AMPA receptor transmission in LID.

5.7.3.3. mGluR

Taking into account the ability of metabotropic glutamate receptors (mGluRs) to finely modulate the excitatory synapse in the brain without blocking fast excitatory neurotransmission, regulation of mGluRs can represent a very intriguing approach for the treatment of LID (Gasparini *et al.*, 2013; Sgambato-Faure and Cenci, 2012). Among the different mGluRs subtypes, mGluR5 is highly expressed in caudate, putamen and basal ganglia, bears a postsynaptic subcellular distribution and represents one of the most promising targets to reduce the excessive glutamatergic transmission which is observed in PD and LID. Different experimental approaches demonstrated an increase of mGluR5-mediated activity in putamen and pallidum associated with LID in both non human primate models of PD and patients (Ouattara *et al.*, 2010a; Samadi *et al.*, 2008). In the last 10 years, specific mGluR5 antagonists or Group II mGluR agonists have been tested for their efficacy in improving motor behaviour in animal models. Overall, these studies indicated that mGluR5 antagonist can reduce LID without affecting L-Dopa therapeutic effect. In particular, several mGluR5

antagonists, such as MPEP, MTEP, fenobam and AFQ056 were found to reduce peak-dose LID while preserving or even potentiating the anti-parkinsonian effect of L-Dopa (Dekundy *et al.*, 2006; Gregoire *et al.*, 2011; Johnston *et al.*, 2010; Maranis *et al.*, 2012; Mela *et al.*, 2007; Morin *et al.*, 2010; Rylander *et al.*, 2009). Importantly, two recent double-blind, placebo-controlled studies confirmed a dose-dependent efficacy of AFQ056, proving the robust anti-dyskinetic effect of this drug without significant worsening of parkinsonian motor symptoms (Berg *et al.*, 2011; Stocchi *et al.*, 2013).

5.7.4. Adenosine receptors

A new class of drugs adenosine A_{2A} receptor antagonists is emerging as a treatment for PD. The basis for the use of these drugs in contrasting the motor symptoms of PD originates from their ability to prolong the therapeutic efficacy of L-Dopa, as demonstrated in both preclinical and clinical studies. Moreover, studies in rodents have shown that A_{2A} receptor antagonists have the ability to contrast dopamine neuron degeneration, which renders this class of drugs particularly suitable for neurodegenerative diseases, such as PD. Because PD requires chronic treatment, the ability of A_{2A} receptor antagonists to revert the dyskinesia induced by dopamine replacement therapy or interrupt its development, is of specific relevance.

The results so far obtained with several A_{2A} receptor antagonists in rodent models of PD suggest that A_{2A} receptor antagonists might have symptomatic therapeutic efficacy in the early stages of PD when motor complication are not yet present (Pinna *et al.*, 2007). In particular, studies in rats suggest that A_{2A} receptor antagonists, when administered alone, may ameliorate initiation of movement, gait, and muscle rigidity whilst simultaneously improving the sensorimotor integration deficits and tremor that characterize PD (Pinna *et al.*, 2007; Salamone *et al.*, 2008; Simola *et al.*, 2004). Moreover, the tests of A_{2A} receptor antagonists in unilaterally 6-OHDA-lesioned rats showed that these drugs potentiate the efficacy of L-Dopa co-administered at a low sub-threshold dose.
In addition to these positive therapeutic effects, A_{2A} receptor antagonists do not demonstrate a dyskinetic profile in both preclinical studies and clinical trials.

In rodents, A_{2A} receptor antagonists do not induce dyskinesia after chronic treatment and do not exacerbate the dyskinesia in rats previously sensitized to L-Dopa (Jones *et al.*, 2013;

Lundblad *et al.*, 2003). As previously described, several studies have shown that chronic administration of L-Dopa to unilaterally 6-OHDA-lesioned rats, besides stimulating AIMs (Lundblad *et al.*, 2002), provoked a sensitization to rotational behaviour, which also represents a model of dyskinesia induced by L-Dopa in humans, since it is only observed after administration of dopamine agonists with high dyskinetic potential (Carta *et al.*, 2008b; Henry *et al.*, 1998; Pinna *et al.*, 2006).

Evaluation of sensitization of rotational behaviour and AIMs after treatment with a full dose of L-Dopa compared with an equipotent combination of a lower dose of L-Dopa plus different A_{2A} receptor antagonists, showed that while the two treatments produced a comparable degree of rotations on the first administration, sensitization of rotational behaviour was observed in response to chronic L-Dopa alone, but not to chronic L-Dopa plus the A_{2A} receptor antagonists SCH58261 or SCH420814 (Hodgson *et al.*, 2009; Pinna *et al.*, 2001; Tronci *et al.*, 2007). These results are supported by studies showing that genetic deletion of the A_{2A} receptor prevents the sensitization of rotational behaviour and AIMs stimulated by L-Dopa in hemiparkinsonian mice (Fredduzzi *et al.*, 2002; Xiao *et al.*, 2006). In agreement, studies by Lundblad et al. (2003) showed that hemiparkinsonian rats treated with the A_{2A} receptor antagonists istradefylline did not develop any AIMs while displaying reduced motor disabilities assessed by a rotarod test (Lundblad *et al.*, 2003). In addition, when istradefylline was chronically administered with L-Dopa at full dose, no modification to the severity of AIMs induced by L-Dopa was observed (Lundblad *et al.*, 2003). These results predicted that co-administration of A_{2A} receptor antagonists and L-Dopa does not prevent or worsen the occurrence of dyskinesia when L-Dopa is given at a full dose, whereas chronic co-administration of A_{2A} receptor antagonists with a low dose of L-Dopa might avoid dyskinesia. The association of the two drugs might therefore represent a treatment with a low dyskinetic potential.

The non-dyskinetic profile of A_{2A} receptor antagonists in L-Dopa-sensitized rats, is consistent with results obtained in MPTP-treated primates chronically treated with istradefylline or preladenant. The A_{2A} receptor antagonists were found not to be prodyskinetic in parkinsonian primates with established dyskinesia in which they relieved motor impairment and did not worsen dyskinesia (Grondin *et al.*, 1999; Hodgson *et al.*, 2010; Kanda *et al.*, 2000). In addition, an attenuation of dyskinesia induced by repeated administration of apomorphine was observed when this drug was administered in combination with an A_{2A} receptor antagonist

(Bibbiani *et al.*, 2003). The previous co-administration of an A_{2A} antagonist was also found to delay the onset of dyskinesia when the same primates were maintained on apomorphine alone (Bibbiani *et al.*, 2003). These latter two results suggest that A_{2A} receptor antagonists might lower the dyskinetic potential of dopamine replacement therapy in specific conditions.

Studies on A_{2A} receptor antagonists as adjuncts to L-Dopa in PD patients with motor fluctuations have generally demonstrated that their addition to a stable L-Dopa regimen is likely to have a reduced dyskinetic liability relative to L-Dopa and, in addition, to reduce "OFF" time and increase "ON" periods (Hauser *et al.*, 2011; Hauser *et al.*, 2008; LeWitt *et al.*, 2008; Mizuno *et al.*, 2010; Stacy *et al.*, 2008). However, A_{2A} receptor antagonists do not contrast dyskinesia when co-administered with L-Dopa (Hauser *et al.*, 2011; Hauser *et al.*, 2008). Moreover, limited clinical data suggest that the addition of an A_{2A} antagonist along with a reduced dose of L-Dopa might maintain an anti-parkinsonian benefit with a lower degree of dyskinesia (Bara-Jimenez *et al.*, 2003). Whether A_{2A} receptor antagonists might reduce the development of dyskinesia has not yet been tested clinically.

The cellular mechanisms at the basis of the findings described above are in relation to the presence of adenosine A_{2A} receptors in several basal ganglia nuclei and to the influence of these receptors on motor activity by acting at different basal ganglia levels (Morelli *et al.*, 2007). An interesting peculiarity of A_{2A} receptors is their selective localization in the indirect GABA/enkephalinergic striatopallidal pathway (Schiffmann *et al.*, 1991), the stimulation of which leads to the inhibition of motor behavior (Ferré *et al.*, 1991; Simola *et al.*, 2004). Interestingly, an increase in A_{2A} receptors in the striatum of 6-OHDA-lesioned rats and of MPTP-treated primates, as well as in PD patients chronically treated with L-Dopa displaying dyskinesia (Brooks *et al.*, 2010; Calon *et al.*, 2004; Pinna *et al.*, 2002; Ramlackhansingh *et al.*, 2011; Tomiyama *et al.*, 2004), might produce a prevailing tone of A_{2A} receptors, the activation of which interferes with motor activity. Therefore, attenuation of the enhanced A_{2A} receptor tone could be one of the factors underlying the positive effects produced by A_{2A} receptor antagonists in PD. Moreover the absence of A_{2A} receptors in the direct GABA/dynorphinergic striatonigral pathway, the efferent pathway more involved in dyskinesia, may favorably influence the non-dyskinetic profile.

There is no evidence to support a direct role of the neuropeptides dynorphin and enkephalin and of the GABA-synthesis enzyme GAD67 in dyskinesia; nevertheless, changes in the

expression of GAD67, dynorphin, and enkephalin have been consistently utilized as a marker of the activity of striatal neurons (Gerfen *et al.*, 1990). Studies in 6-OHDA-lesioned rats demonstrate that dopamine denervation is associated with an elevation of striatal GAD67 and enkephalin mRNA levels and with a decrease in dynorphin mRNA levels (Carta *et al.*, 2002; Lundblad *et al.*, 2003). Interestingly, while chronic-intermittent L-Dopa dyskinetic treatment increased the striatal levels of GAD67, dynorphin, and enkephalin mRNA in the lesioned side, chronic-intermittent combined administration of an equi-effective dose of an A_{2A} receptor antagonist plus L-Dopa, besides resulting in a stable motor response, did not produce any significant modification in GAD67, dynorphin, or enkephalin mRNA in the intact striatum compared with vehicle-treated rats (Carta *et al.*, 2002; Gerfen *et al.*, 1990; Lundblad *et al.*, 2003). Therefore, a combination of A_{2A} receptor antagonists together with L-Dopa may produce attenuation of the neuroplastic modifications in the striatal functions that underlie dyskinesia.

Several conclusions can be drawn from these studies. Firstly, A_{2A} receptor antagonists have a reduced dyskinetic liability relative to L-Dopa, but do not contrast dyskinesia induced by L-Dopa. Secondly, they may delay L-Dopa-induced dyskinesia without affecting its therapeutic efficacy if L-Dopa-sparing doses are used. Therefore A_{2A} receptor antagonists may be envisioned as a class of non-dopaminergic drugs that might act positively on PD motor symptoms and potentiate L-Dopa therapeutic efficacy without having dyskinetic potential. Association of A_{2A} receptor antagonists to antidyskinetic drugs, which while reducing dyskinesia also decrease the therapeutic efficacy of L-Dopa, may therefore offer a new prospective for the treatment of dyskinesia in PD.

5.7.5. Dysregulation of homologous desensitization

The deregulation of DA-mediated signalling manifests itself as strongly enhanced responsiveness to dopaminergic stimulation both at the behavioural and signalling levels via all major striatal DA receptor subtypes (Brown *et al.*, 2005; Bychkov *et al.*, 2007; Cai *et al.*, 2000; Corvol *et al.*, 2004; Gerfen, 2000; Gerfen *et al.*, 2002a; Pifl *et al.*, 1992a; Pifl *et al.*, 1992b; Ravenscroft *et al.*, 2004; Sgambato-Faure *et al.*, 2005; Tong *et al.*, 2004; Ungerstedt, 1971b). Although the mechanism of dopaminergic supersensitivity is undoubtedly complex,

deregulation of the receptor desensitization machinery is likely to play an important role in LID.

DA receptors belong to the superfamily of G protein-coupled receptors (GPCR) that transmit signals in response to a wide variety of stimuli via an uniform mechanism involving coupling of liganded receptors to heterotrimeric G proteins followed by GTP-GDP exchange on α-subunit and dissociation of α-subunit from the ßγ-dimer, both of which activate or modulate effectors (Rasmussen *et al.*, 2011). Activation of a GPCR by an agonist initiates G protein-mediated signalling and at the same time triggers a shutdown mechanism termed homologous desensitization, or desensitization of the receptors that are being activated. The classic model of homologous desensitization of GPCRs posits that agonist-activated receptors are first phosphorylated by G protein-coupled receptor kinases (GRKs) [reviewed in (Gurevich *et al.*, 2012)]. GRKs specifically recognize receptor conformations conducive to G protein binding and, like G proteins, directly bind active receptors (Huang and Tesmer, 2011). Since this interaction of a GRK with an activated receptor activates the kinase (Palczewski *et al.*, 1991), GRKs are selective towards activated GPCRs. The receptor phosphorylation promotes high-affinity binding of uncoupling proteins arrestins. Arrestin shields the cytoplasmic surface of the receptor, precluding further G protein activation (Krupnick *et al.*, 1997; Wilden, 1995). Arrestin binding also promotes receptor internalization by virtue of direct arrestin interaction with clathrin and AP-2, the main components of the coated pit (Goodman *et al.*, 1996; Laporte *et al.*, 1999), leading to the receptor resensitization and recycling or, in some cases, down-regulation (Morrison *et al.*, 1996; Pan *et al.*, 2003; Wu *et al.*, 2008) (**Figure 5A**).

GRK phosphorylation reduces receptor coupling to G proteins (Wilden, 1995), but does not eliminate it. The full signal shutoff is accomplished by the binding of an arrestin to active phosphorylated GPCR (Attramadal *et al.*, 1992; Krupnick *et al.*, 1997; Lohse *et al.*, 1992; Lohse *et al.*, 1990; Wilden, 1995). Arrestin requires more than one phosphate attached to a receptor for high affinity binding (Vishnivetskiy *et al.*, 2007). Arrestins have been shown to compete with G proteins for active GPCRs (Krupnick *et al.*, 1997; Wilden, 1995). However, because the receptor needs to be phosphorylated multiple times before arrestin can bind with high affinity, G protein has a time window with a clear advantage over arrestin, when it can be activated and transmit the signal before the shutoff is complete. For example, light-

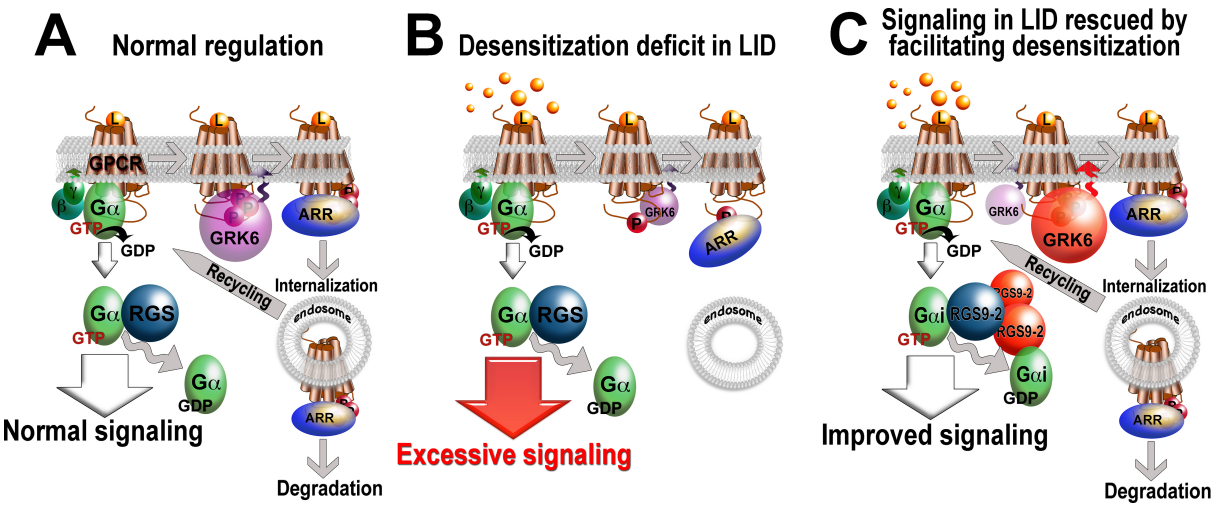

Figure 5. Receptor desensitization mechanisms play a critical role in signaling abnormalities associated with L-Dopa-induced dyskinesia. (A) The G protein-coupled receptor (GPCR) upon activation by a ligand (L) promotes an exchange of GDP for GTP on cognate heterotrimeric G protein and dissociation of Gα subunit from ßγ-dimer. Active GTP-liganded Gα activates downstream signalling pathways until deactivated due to GTP hydrolysis to GDP by intrinsic GTPase activity of Gα. GTPase activity is enhanced by Regulators of G protein Signalling (RGS) proteins that thus accelerate G protein deactivation. Active receptor is recognized and phosphorylated by a G protein-coupled receptor kinase (GRK; GRK6 isoform is shown). Receptor phosphorylation promotes high-affinity binding of arrestin (ARR) that precludes further G protein interaction with the receptor and promotes receptor internalization via coated pits. Receptor internalization is the starting point for the receptor dephosphorylation in endosomes and, in most cases, recycling back to the plasma membrane. In case of persistent receptor activation, internalization could be followed by receptor degradation and downregulation. (**B**) In the condition of L-Dopa-induced dyskinesia (LID), intense stimulation of dopamine receptors with an abundant ligand (dopamine produced from L-Dopa) coupled with defective desensitization results in excessive signalling. Desensitization deficit could be brought about by a reduction in the concentration of GRKs caused by the loss of dopamine or could be due to insufficiency of the capacity of the desensitization machinery relative to the demand. (**C**) Excessive signalling in LID could be improved by supplying exogenous GRK6 to compensate for the loss of GRK6 in the dopamine-depleted striatum or by expressing additional striatum-specific RGS9-2, although there is no appreciable loss of RGS9-2 in LID.

activated rhodopsin, a prototypical class A GPCR, is capable of sequentially activating dozens of G protein molecules (Leskov *et al.*, 2000). The receptor phosphorylation by GRK is the rate-limiting step in the homologous desensitization process (Violin *et al.*, 2008), and GRK concentration in cells strongly influences the rate and extent of receptor desensitization, as well as the duration and intensity of G protein-mediated signalling (Gainetdinov *et al.*, 2003; Gainetdinov *et al.*, 1999; Gainetdinov *et al.*, 2004; Iaccarino *et al.*, 1998; Kim *et al.*, 2001; Menard *et al.*, 1997; Pan *et al.*, 2003; Willets *et al.*, 2004; Willets *et al.*, 1999). Active GTP-liganded α-subunits of G proteins are in their turn deactivated via hydrolysis of GTP to GDP by intrinsic GTPase activity of the α-subunits. That activity is enhanced by GTPase activating proteins (GAPs) that accelerate G protein deactivation and reduce signalling. The major class of GAPs are Regulators of G protein Signalling (RGS) (Ross and Wilkie, 2000; Siderovski *et al.*, 1996; Siderovski and Willard, 2005). When the receptor is uncoupled from G protein via GRK-arrestin-dependent desensitization, the signalling is sustained by remaining active G proteins. RGS proteins, by facilitating G protein deactivation, promote complete signal shutoff (**Figure 5A**). The cellular concentration of RGSs is a critical determinant of the signalling intensity. In some systems, RGSs and not GRKs are rate-limiting for the overall signal shutoff (Krispel *et al.*, 2006). Any deregulation of this complex well-orchestrated mechanism of termination of the GPCR signalling would result in a profound enhancement of signal duration and/or intensity and is likely to bring about multiple behavioural deficits.

Mammals express seven GRK subtypes, with two isoforms, GRK1 and GRK7, being confined to the retinal photoreceptors and one, GRK4, - largely to testes (Gurevich *et al.*, 2012; Mushegian *et al.*, 2012). Four isoforms, GRK2, 3, 5, and 6, are ubiquitously expressed throughout the brain (Ahmed *et al.*, 2007; Ahmed *et al.*, 2008; Bychkov *et al.*, 2010; Bychkov *et al.*, 2011; Bychkov *et al.*, 2013; Bychkov *et al.*, 2008). Since the number of non-visual GRKs is limited and much lower than the number of ~ 700 mammalian GPCRs they serve, it is generally assumed that each isoform phosphorylates numerous GPCRs. However, studies in GRK knockout mice brought forward evidence of *in vivo* receptor specificity of GRKs [see discussion in (Gurevich *et al.*, 2012)]. Furthermore, recent evidence of differential functional consequence of the receptor phosphorylation by different GRKs [the ”barcode” concept) (Kim *et al.*, 2005; Liggett, 2011; Nobles *et al.*, 2011; Ren *et al.*, 2005; Zidar *et al.*, 2009)] strongly suggests that GRK isoforms are not interchangeable, but each has a defined function. The receptor specificity and functional role of GRKs *in vivo* remains to be elucidated [for in depth discussion see (Gurevich *et al.*, 2012)]. The human and rodent striatum expresses all four

ubiquitous non-visual GRKs (Ahmed *et al.*, 2010; Ahmed *et al.*, 2007; Ahmed *et al.*, 2008; Bychkov *et al.*, 2013; Bychkov *et al.*, 2008). In the rat, GRKs 2 and 5 are equally expressed in the direct and indirect pathway medium spiny neurons, but GRK2 is highly enriched in cholinergic interneurons, as compared to the output neurons, whereas GRK5 is expressed at similar level is both (Bychkov *et al.*, 2013). Unfortunately, the expression pattern of GRK6, the highest expressed GRK in the rodent striatum, is yet undefined. Experiments with GRK knockout mice demonstrated that mice lacking GRK6 were supersensitive to behavioural effects of dopaminergic drugs, whereas mice lacking the closest relative of GRK6, GRK5, were not (Gainetdinov *et al.*, 2003; Gainetdinov *et al.*, 1999; Gainetdinov *et al.*, 2004). The data strongly suggest that GRK6 is primarily responsible for desensitization of DA receptors. Furthermore, the data support the notion that loss of GRK6 results in enhanced responsiveness of DA receptors to dopaminergic stimulation.

When dopaminergic neurons degenerate in PD or in animal models of PD, striatal dopaminoreceptive neurons put into place a number of adaptive mechanisms aimed at maintaining the failing signalling (Bezard and Gross, 1998). One effective adaptive response would be a reduction in the level of GRKs. Indeed, in hemiparkinsonian rats, the level of GRKs in the lesioned striatum is decreased, as compared to the intact side, most noticeably that of GRK6 and GRK3 (Ahmed *et al.*, 2010; Ahmed *et al.*, 2007). Such effect could be considered adaptive, because it counteracts the effect of the dopaminergic lesion, allowing, due to resulting DA receptor supersensitivity, for the signal transmission even with the grossly reduced concentration of DA. Importantly, this reduction in the GRK concentration was not reversed by chronic L-Dopa treatment (Ahmed *et al.*, 2010; Ahmed *et al.*, 2007). GRK6, presumably the main isoform regulating DA receptors, was consistently reduced by DA depletion across striatal subdivisions. The decrease of GRK6A, the splice variant most abundant in the rat brain at the mRNA level (Firsov and Elalouf, 1997) (the protein levels were never compared), reached ~40%, whereas GRK6B splice variant was only marginally reduced (Ahmed *et al.*, 2010). The GRK concentration also tended to be lower in postmortem striatal samples from human PD patients without dementia (Bychkov *et al.*, 2008), which might be the result of years of L-Dopa treatment and associated with LID, since the samples were mostly from end-stage patients. Interestingly, in MPTP-treated parkinsonian drug-naïve monkeys, GRKs, particularly GRKs 2 and 6, were elevated as compared to control, and chronic L-Dopa reduced the expression to normal in both non-dyskinetic and overtly dyskinetic animals (Bezard *et al.*, 2005). It is conceivable that in this case the increase in the

GRK concentration was a part of the pathological process aggravating signalling deficiency. The L-Dopa treatment reverted the defect by reducing the GRK concentration, but at the same time the GRK availability could have become grossly insufficient during high signalling periods at the peak L-DOPA concentration. Overall, the background of low GRK availability relative to the demand at the time of high DA concentration generated from peak-dose L-Dopa is likely to be a contributing factor to signalling abnormalities associated with peak-dose LID (**Figure 5B**).

This idea was tested by studying the effect of *in vivo* knockdown of GRK6 in the lesioned striatum of hemiparkinsonian rats using lentivirally-delivered miRNA (Ahmed *et al.*, 2010). The reduction in the GRK6 achieved by such knockdown was slightly less than 40% for both GRK6A and GRK6B proteins. The GRK6 knockdown strongly enhanced the frequency of L-Dopa-induced contralateral rotations and promoted behavioural sensitization to L-Dopa, the phenomenon relevant for LID. Furthermore, rats with reduced GRK6 concentration demonstrated increased frequency of AIMs (Ahmed *et al.*, 2010). These data further support the notion that a deficit in GRK availability, specifically that of GRK6, leads to defective desensitization, enhanced signalling, and ultimately, promotes LID-like behaviour. The fact that a relatively modest loss of GRK6 was sufficient to significantly affect behaviour underscores the critical contribution of the GRK-dependent regulation of the dopaminergic signalling to LID.

If reduced GRK concentration aggravated LID, then increased GRK availability should ameliorate it. The study employing lentivirus-mediated overexpression of GRK6A the DA-depleted striatum showed that increased GRK6 concentration resulted in reduced frequency of L-Dopa-induced rotations and lower AIMs scores (Ahmed *et al.*, 2010). The behavioural improvement was accompanied by amelioration of molecular hallmarks of LID: characteristic upregulation of prodynorphin and preproenkephalin mRNA and the D3 receptor concentration in the caudate-putamen were all significantly reduced in the GRK6-expressing rats as compared to the GFP-expressing control. Furthermore, lentiviral overexpression of GRK6 in the putamen of MPTP-lesioned monkeys rendered dyskinetic by chronic L-Dopa treatment significantly ameliorated peak-dose LID (Ahmed *et al.*, 2010). As in the case of the rodent model of LID, the behavioural improvement was accompanied by a reduction in the level of prodynorphin mRNA, which was elevated in L-Dopa-treated animals. Thus, the data in both the rodent and monkey models of LID support the anti-LID potential of GRK6 (**Figure 5C**).

GRK6 is likely to alter the DA-dependent behaviour by facilitating desensitization of DA receptors. This notion is supported by the fact that trafficking of the D1 DA receptor is markedly improved in the lesioned striatum of rats expressing GRK6, whereas the D2 receptor was unaffected (Ahmed *et al.*, 2010). These data appear to be inconsistent with the previous finding in GRK6 knockout mice that behavioural supersensitivity to psychostimulants in these animals to modified signalling via the D2 but not the D1 receptor (Gainetdinov *et al.*, 2003). However, DA depletion and subsequent LID development in the course of L-Dopa treatment precipitates a dramatic change in the function of striatal DA receptors that become supersensitive to dopaminergic stimulation [(Brown *et al.*, 2005; Bychkov *et al.*, 2007; Cai *et al.*, 2000; Corvol *et al.*, 2004; Gerfen, 2000; Gerfen *et al.*, 2002a; Pifl *et al.*, 1992a; Pifl *et al.*, 1992b; Ravenscroft *et al.*, 2004; Sgambato-Faure *et al.*, 2005; Tong *et al.*, 2004; Ungerstedt, 1971b) see also (Gurevich and Gurevich, 2010) and references therein]. It is generally believed that both major receptor subtypes contribute to LID, but the D1 receptor seems to play a particularly important role (Aubert *et al.*, 2005; Berthet *et al.*, 2009; Guigoni *et al.*, 2005a; Guigoni *et al.*, 2007), and multiple aberrations in D1 signalling are readily detectable in the brain of dyskinetic animals (Aubert *et al.*, 2005; Berthet *et al.*, 2009; Gerfen, 2000; Gerfen *et al.*, 1990; Gerfen *et al.*, 1995; Gerfen *et al.*, 1991; Gerfen *et al.*, 2002a; Guigoni *et al.*, 2007). In the dyskinetic monkeys, transgenic expression of GRK6 reduced LID caused by either the selective D1 agonist SKF 38393 or the D2 agonist ropinirole, indicating that its anti-LID affect was mediated via both receptor subtypes (Ahmed *et al.*, 2010). The suppression by GRK6 of the L-Dopa-induced upregulation of prodynorphin and D3 receptor mRNA in hemiparkinsonian rats and prodynorphin elevation in parkinsonian monkeys also support the notion of GRK6 acting at the D1 DA receptor, since both effects are attributed to the enhanced D1 receptor signalling (Bordet *et al.*, 1997; Gerfen *et al.*, 1990; Gerfen *et al.*, 1991). Although no GRK6-induced increase in the D2 receptor internalization was detected, GRK6 reduced the upregulation of preproenkephalin mRNA expressed in D2 receptor-bearing neurons (Gerfen *et al.*, 1990; Gerfen *et al.*, 1991; Le Moine and Bloch, 1995; Morissette *et al.*, 1997), which suggests a GRK6 effect at the D2 DA receptor. Thus, the data in the rodent and monkey models of LID collectively point to the involvement of both D1 and D2 receptors in the anti-LID action of GRK6.

However, studies in living animals cannot prove that the effect is direct. Since the change in the D1 receptor trafficking in the rat model was observed, this would suggest a direct GRK6-

dependent phosphorylation of the D1 receptor followed by arrestin binding and intracellular trafficking. The lack of a similar effect on the D2 receptor leaves room for doubt. However, receptor desensitization may not necessarily be accompanied by internalization (Pan *et al.*, 2003), and trafficking measures could be underestimating the degree of desensitization. Alternatively, D2 receptors could be less affected by GRK6, since they are known to be resistant to desensitization (Kim *et al.*, 2001; Tiberi *et al.*, 1996). The data in the monkey model bears out this suggestion. When the animals were treated with selective D1 or D2/D3 agonists instead of L-Dopa, transgenic GRK6 not only suppressed LID but also shortened the overall duration of their effects, including the antiparkinsonian activity. This mode of action is likely reflective of faster and more profound receptor desensitization due to increased GRK6 availability. GRK6 had only a marginal effect on the duration of D2-mediated effects, whereas it substantially shortened that of the D1 agonist, which again supports the notion of the D1 receptor as the prime target of GRK6. It is important to bear in mind that striatal neurons express other non-DA GPCRs that modulate LID and could be affected by GRK6. It is possible that the effect of GRK6 on the D2-dependent signalling is in fact indirectly mediated by other receptors, such as, for example, the adenosine A2 receptor. The inactivation or inhibition of the A2 receptor is known to ameliorate LID and/or provides antiparkinsonian benefits in parkinsonian animals and humans (Fredduzzi *et al.*, 2002; Lundblad *et al.*, 2003; Xiao *et al.*, 2006). GRK6-dependent desensitization of the A2 receptor would mimic its inactivation bringing about anti-LID and signalling benefits associated with reduced A2 signalling. The inhibition of the lesion-induced upregulation of enkephalin by GRK6 may be the result of such suppression of A2 receptor activity, similarly to the action of the A2 antagonist KW-6002 (Lundblad *et al.*, 2003).

The biggest stumbling block in the development of viable anti-LID therapies has been separating therapeutic and dyskinetic effects of L-Dopa. Both functions of the drug are mediated by DA receptors, and over the years of therapy the antiparkinsonian and dyskinetic effects become so intertwined, that reducing LID may mean loosing antiparkinsonian effect as well. Remarkably, GRK6 suppresses LID in dyskinetic monkeys without compromising the antiparkinsonian effects of L-Dopa. In fact, GRK6 prolongs the antiparkinsonian effect, especially at the lower L-Dopa dose. The duration of the antiparkinsonian effect of the half-dose in GRK6-expressing animals was even slightly longer than that of the full L-Dopa dose in controls. Importantly, the additional time afforded by GRK6 was LID-free (Ahmed *et al.*, 2010). Mechanistically, preservation of the antiparkinsonian activity coupled with reduced

LID likely stems, at least, in part, from GRK selectivity towards active GPCRs (Boguth *et al.*, 2010; Huang *et al.*, 2011a; Huang and Tesmer, 2011; Huang *et al.*, 2009). The receptor must be activated for GRK to bind and phosphorylate it, initiating the shutdown process. Therefore, the signal will go through, and a certain number of active G proteins will be generated before the receptor is uncoupled from G proteins via GRK/arrestin-mediated desensitization. This initial signalling may be sufficient for the antiparkinsonian effect but receptor desensitization process prevents it from rising high enough to cause LID. This is hardly surprising, because the receptor desensitization machinery is designed to achieve precisely this effect: to limit the duration and intensity of the signal following the receptor activation but not to prevent the signalling event. Additionally, GRK6-dependent rebalancing of the striatal circuitry may also play a beneficial role. The fact that GRK6 extended the antiparkinsonian effect of L-Dopa while shortening that of both D1 and D2 selective agonists may due to the action of L-Dopa-derived DA at both D1 and D2 receptors. If GRK6 mostly desensitized D1 receptors, it would shift the overall signalling balance in favor of the D2-mediated signalling, reducing the D1-dependent LID but sustaining the beneficial effect through still active D2 receptors. Thus, the receptor desensitization mechanism seems like a perfect target when there is a need to rebalance the runaway signalling. Indeed, the data in the monkey model of PD prove that targeting the receptor desensitization machinery for anti-LID therapy may help to reach an elusive goal of controlling LID without sacrificing the antiparkinsonian benefits of L-Dopa. Furthermore, with time PD patients experience a reduction in duration of L-Dopa antiparkinsonian effect. Such shorter effect together with uncertain and sometimes absent effect of the drug are referred to as motor fluctuations. Motor fluctuations is another factor, in addition to LID, that severely limits the efficacy of L-Dopa therapy in PD. Unfortunately, little is known about molecular mechanisms of these effects. It is conceivable that increased duration of L-Dopa therapeutic effect in parkinsonian monkeys expressing transgenic GRK6 is indicative of the GRK6 potential to combat motor fluctuations as well as LID.

When DA receptors are completely desensitized with arrestins preventing further coupling to G proteins, previously generated active G proteins may still persist and activate downstream targets. The G proteins deactivation is accelerated by RGSs, and RGS availability and function is an important determinant of the signal intensity and duration. Therefore, it is conceivable that RGS function is perturbed in LID contributing to the deregulation of the DA receptor signalling. The RGS family is large and diverse (Ross and Wilkie, 2000), with many members expressed in striatal neurons (Gold *et al.*, 2007a; Gold *et al.*, 1997). The RGS9-2

isoform is highly enriched in the striatum in comparison with other brain structures (Gold *et al.*, 2007a; Gold *et al.*, 1997; Granneman *et al.*, 1998; Kovoor *et al.*, 2005; Rahman *et al.*, 1999; Rahman *et al.*, 2003). The concentration of multiple RGS proteins is responsive to changes in the dopaminergic environment (Ding *et al.*, 2006; Geurts *et al.*, 2002; Geurts *et al.*, 2003). However, neither DA depletion nor subsequent L-Dopa treatment altered the expression of RGS9-2 in the monkey striatum (Gold *et al.*, 2007a). No changes were seen in other RGS proteins abundant in the striatum: RGS2, 7, 4 or in the level of RGS anchoring protein Gb5. Nevertheless, viral upregulation of RGS9-2 resulted in reduced LID coupled with preservation of the antiparkinsonian effect of L-Dopa in dyskinetic MPTP-lesioned monkeys and in the reduction in AIMs scores in hemiparkinsonian rats (Gold *et al.*, 2007a). Conversely, mice lacking RGS9-2 were more sensitive to LID-inducing effect of L-Dopa demonstrating higher AIMs scores than wild type mice (Gold *et al.*, 2007a) (**Figure 5C**). Importantly, the magnitude of the RGS9-2-dependent effects was considerably smaller than that observed in the experiments with GRK6. Thus, complete elimination of RGS9-2 in knockout mice causes a relatively minor increase in the AIMs score, whereas a modest less than 40% knockdown of GRK6 yielded a robust long-term increase in AIMs score as well as suppressed the sensitization process (Ahmed *et al.*, 2010). The likely reason is that RGS9-2 selectively binds to and accelerates deactivation of Gαi, but not Gαs/olf (Rahman *et al.*, 2003; Ross and Wilkie, 2000). Thus, RGS9-2 quenches the signalling via the Gi-coupled D2 receptor but not via Gs/olf-coupled D1 receptor, whereas GRK6 apparently acts via both DA receptors. Since the D1 receptor deregulation is believed to make the leading contribution to molecular mechanisms responsible for LID (Aubert *et al.*, 2005; Berthet *et al.*, 2009; Guigoni *et al.*, 2005a; Guigoni *et al.*, 2007), targeting exclusively the D2 receptor is less effective than targeting both. Nevertheless, facilitating the RGS9-2-dependent quenching of the Gi-dependent signalling offered substantial anti-LID benefits in the monkey model of LID, proving that RGS-dependent desensitization of the DA receptor signalling is a critical component of the signalling homeostasis in striatal neurons, and its deregulation is likely to be a part of LID pathophysiology.

To summarize, known molecular mechanism of action of GRKs and arrestins suggests that these proteins play key role in neuronal adaptations, including changes in signalling caused by DA depletion and subsequent L-Dopa therapy. The role of RGS proteins in the signalling aberrations associated with LID also deserves attention. Unfortunately, mechanistic information regarding precise role of GRKs, arrestins, or RGSs in the physiological processes

associated with these signalling adaptations is currently very limited. GRK6 appears to be the best therapeutic target with proven efficacy in both rodent and monkey models of LID. Furthermore, as a kinase, GRK6 is a "druggable" target. As a group, kinases are second only to GPCRs as drug targets (Cohen, 2002; Melnikova and Golden, 2004). At the moment, there are no drugs selectively aimed at GRK6 or even at the whole GRK4 subfamily, which includes GRKs 4, 5, and 6. Furthermore, for this particular purpose an activator rather than inhibitor would be needed. To the best of our knowledge, no drug that enhances the activity of any GRK has been found. This is perhaps not surprising, given the mode of the drug discovery effort for kinases that so far targeted mostly the kinase domain in search for kinase inhibitors (Fischer, 2004; Ma *et al.*, 2008; Melnikova and Golden, 2004; von Ahsen and Bömer, 2005). Only recently the approach has been expended to incorporate allosteric type regulators that could act as activators as well as inhibitors (Eglen and Reisine, 2011; Simpson *et al.*, 2009). For GRKs, targeting the GRK-receptor interface offers the best opportunity to find isoform-selective modulators enhancing or inhibiting their activity, although this is by no means a trivial task. Alternatively, drugs regulating the expression, and/or stability of GRKs, arrestins, or RGSs could be developed. Pathway biased agonists for DA receptors that preferentially engage GRK-mediated receptor phosphorylation and arrestin binding are becoming available (Shukla *et al.*, 2011; Violin and Lefkowitz, 2007; Zidar *et al.*, 2009) and can be further developed. Since the receptor desensitization system is a natural mechanism designed to adjust GPCR responsiveness to the intensity and duration of receptor stimulation, manipulation of its capacity is likely to yield a precisely fine-tuned regulation of the signalling that could be exploited for efficacious anti-LID therapy.

6. Other changes in basal ganglia and beyond

6.1. Cholinergic receptors

6.1.1. Nicotine administration reduced LIDs in parkinsonian animal models

Emerging work indicates that the nicotinic cholinergic system plays a role in LID. Evidence for this idea is supported by results using several parkinsonian animal models. Studies in 6-OHDA-lesioned rats or MPTP-lesioned mice showed that nicotine reduced AIMs up to 60% (**Figure 6**), including axial, oral and forelimb AIMs (Bordia *et al.*, 2008; Bordia *et al.*, 2010; Huang *et al.*, 2011b; Huang *et al.*, 2011c; Quik *et al.*, 2012a). The nicotine-induced decline in

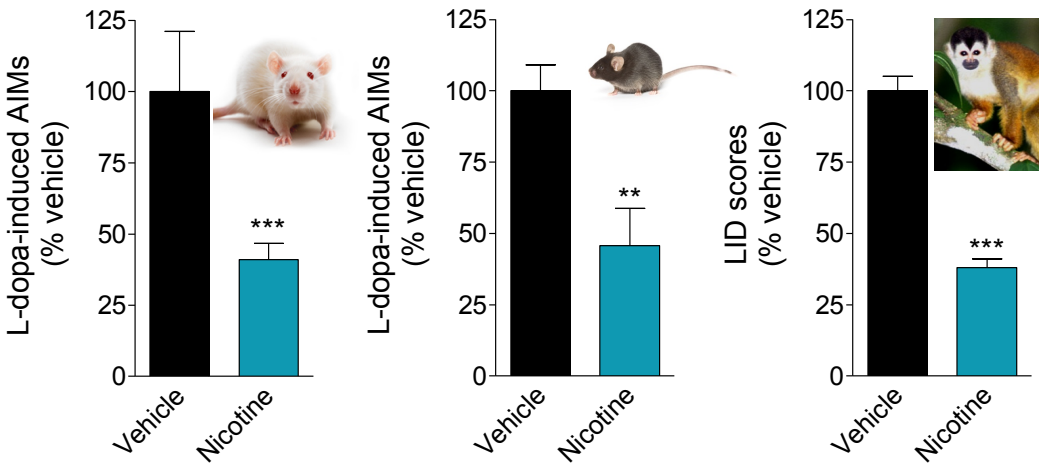

Figure 6. Nicotine treatment reduces LIDs across species. Nicotine consistently reduces LIDs in parkinsonian rats, mice and monkeys with no worsening of parkinsonism on or off L-dopa. Taken in modified form from (Bordia *et al.*, 2008; Huang *et al.*, 2011b; Quik *et al.*, 2007a; Quik *et al.*, 2013d). Notably, the maximal decline in LIDs ranges between 60-70% across species. Significance of difference from vehicle-treated animals, **p < 0.01, ***p < 0.001. Values are the mean ± SEM of 5-6 animals.

nAChR subtype deleted	Type of nAChR subunit knockout mouse	Baseline L-dopa-induced AIMs in knockout	Nicotine still decreases AIMs in knockout
α4β2* and α6β2*	β2 (-/-)	Reduced	No
α6β2*	α6 (-/-)	Reduced	No
α4β2*	α4 (-/-)	Unaffected	No
α7	α7 (-/-)	Enhanced	Yes

Table 1.
α4β2*, α6β2* and α7 nAChRs modulate expression of L-dopa-induced AIMs. Data from nAChR knockout mice show that nAChR subtypes may modify the level of AIMs (baseline) and/or the ability of nicotine to reduce L-dopa-induced AIMs (Huang *et al.*, 2011b; Quik *et al.*, 2013b; Quik *et al.*, 2012a).

L-Dopa-induced AIMs persisted with long term treatment (months) and was observed with varying modes of nicotine treatment including systemic injection, slow-release minipumps or via the drinking water. Notably, there was no worsening of parkinsonism with nicotine administration.

In addition, the effect of nicotine on LIDs has been investigated in MPTP-lesioned non human primates. Monkeys were given nicotine in the drinking water, a paradigm that readily lends itself to long term treatment. Nicotine maximally reduced LIDs with 60-70% declines in both peak and total LIDs after several weeks **(Figure 6)** (Quik *et al.*, 2007a; Quik *et al.*, 2013c; Quik *et al.*, 2013d). Again, there was no effect on parkinsonism. Nicotine led to a similar reduction in LIDs whether it was given to L-Dopa naïve monkeys or animals with established LIDs; thus, nicotine can be used prophylactically or to reduce existing LIDs (Quik *et al.*, 2007a; Quik *et al.*, 2013d). There was no tolerance to the nicotine-induced decline in LIDs for the entire study duration (up to 1 year). This is an important point as PD patients generally require life-long treatment with L-Dopa (Quik *et al.*, 2007a; Quik *et al.*, 2013d). Nicotine's antidyskinetic effect remained for several weeks after drug discontinuation, suggesting that long term molecular changes underlie the improvement. Studies with varying degrees of nigrostriatal damage showed that nicotine best reduced LIDs in animal models with a moderate nigrostriatal loss (Bordia *et al.*, 2010; Quik *et al.*, 2013c), suggesting it may not be that effective in late-stage PD. These studies in animal models have been extended to the clinic; a small trial in PD patients showed that oral nicotine administration reduced various components of LIDs (http://www.neuraltus.com/pages/news_rel12_03_10.html). Altogether, these findings suggest that nicotine may be useful for the treatment of LIDs in PD patients.

6.1.2. Nicotine decreases LIDs by acting at nACHRs

Nicotine generally exerts its CNS effects by acting at nicotinic acetylcholine receptors (nAChRs), which are ligand-gated ion channel composed of five membrane-spanning subunits. The primary subtypes in mammalian brain are heteromeric $\beta 2$* and homomeric $\alpha 7$ receptors, with the asterisk indicating the presence of other nAChR subunits in the receptor complex (Albuquerque *et al.*, 2009; Millar and Gotti, 2009; Quik and Wonnacott, 2011). The most populous subtypes in the basal ganglia are the $\alpha 4 \beta 2$* and $\alpha 6 \beta 2$* nAChRs, with $\alpha 7$ nAChRs expressed to a lesser degree (Albuquerque *et al.*, 2009; Millar and Gotti, 2009; Quik and Wonnacott, 2011). Two approaches to investigate the nAChR subtypes that are involved

in the regulation of LIDs include the use of genetically modified mice and nAChR subtype drugs, as described below.

6.1.2.1. nAChR subunit null mutant mice

Studies with nAChR knockout mice indicate that nicotine's antidyskinetic effect is mediated via multiple subtypes as summarized in **Table 1**. $\alpha 6$ nAChR subunit knockout mice, which lack $\alpha 6\beta 2$* nAChRs, had reduced baseline L-Dopa-induced AIMs (Quik *et al.*, 2012a). Furthermore, there was no decline in remaining AIMs with nicotine treatment in $\alpha 6$ nAChR knockout mice compared to wild type mice (Quik *et al.*, 2012a). Thus, nAChRs expressing the $\alpha 6$ subunit are important for both the generation of L-Dopa-induced AIMs and the antidyskinetic effect of nicotine. Nicotine also did not reduce L-Dopa-induced AIMs in α4 nAChR null mutant mice, although baseline AIM scores were unaffected in these mice (Quik *et al.*, 2013b). These data indicate that both $\alpha 6\beta 2$* and $\alpha 4\beta 2$* nAChRs regulate AIMs although in a somewhat different fashion.

Experiments with $\alpha 7$ nAChR null mutant mice showed that these receptors modulated L-Dopa-induced AIMs in a manner distinct from that by $\alpha 4\beta 2$* and $\alpha 6\beta 2$* nAChRs. First, there was an increase in baseline L-Dopa-induced AIMs in $\alpha 7$ nAChR knockout mice, suggesting that α7 nAChRs have an inhibitory impact (Zhang *et al.*, 2013). Second, nicotine treatment still decreased AIMs in $\alpha 7$ nAChR knockout mice. The variable mode of regulation by $\beta 2$* and $\alpha 7$ nAChRs may arise because of their differential expression, molecular properties and functional characteristics. For instance, α7 nAChRs are more permeable to calcium, desensitize more rapidly and are linked to alternate intracellular signalling pathways compared to β2* nAChRs (Changeux, 2010; Giniatullin *et al.*, 2005; Picciotto *et al.*, 2008; Quik *et al.*, 2012b; Wonnacott *et al.*, 2005).

In summary, studies with genetically modified mice indicate that $\alpha 4\beta 2$*, $\alpha 6\beta 2$* and $\alpha 7$ nAChRs are all involved in the occurrence of LIDs, although in distinct manners (**Table 1**).

6.1.2.2. Pharmacological studies

The data with nAChR knockout mice led to studies testing the effect of nAChR drugs on LIDs. The general nAChR agonist varenicline reduced dyskinesias in both L-Dopa-treated rats and monkeys, providing proof of principle that the effect of nicotine was nAChR-mediated (Huang *et al.*, 2011c; Zhang *et al.*, 2013). A role for β2* nAChRs is suggested from work with A-85380 and a series of Targacept compounds, which all reduced LIDs in 6-OHDA-lesioned rats (Huang *et al.*, 2011c; Quik *et al.*, 2013a). In addition, the β2* nAChR agonist TC-8831 reduced LIDs in parkinsonian macaques and squirrel monkeys, with no worsening of parkinsonism (Johnston *et al.*, 2013; Quik *et al.*, 2013d). The precise contribution of the α4β2* and α6β2* nAChRs on LIDs has not been possible using a pharmacological approach since available drugs act at both receptor subtypes. The role of α7 nAChR drugs has not yet been investigated.

6.1.3. Mechanism of the nAChR-mediated decline in dyskinesias

The somewhat paradoxical finding that nAChR agonists and the antagonist mecamylamine both reduce L-Dopa-induced AIMs to a similar extent in parkinsonian rats has led to the suggestion that nAChR agonists reduce AIMs via nAChR desensitization (Bordia *et al.*, 2010). This molecular event leads to a functional blockade similar to that observed with antagonists (Buccafusco *et al.*, 2009; Corringer *et al.*, 2006; Picciotto *et al.*, 2008). Long term nicotine treatment also downregulated α6β2* nAChRs (Lai *et al.*, 2005). Thus both nAChR-induced desensitization and downregulation may underlie the nicotine-mediated reduction in LIDs.

LIDs are thought to arise because of L-Dopa-mediated transient increases in striatal dopamine release, which leads to disproportionate dopaminergic stimulation (Carta and Bezard, 2011; Cenci, 2007a; Fisone and Bezard, 2011; Lindgren *et al.*, 2010). Long term nicotine treatment has been shown to reduce striatal nAChR-mediated dopamine release (Bordia *et al.*, 2013). These combined findings suggest that chronic nicotine treatment desensitizes and/or downregulates nAChRs, with a consequent decline in striatal dopamine release and subsequent improvement in L-Dopa-induced AIMs (Bordia *et al.*, 2013).

With respect to the localization of the nAChRs involved in regulating LIDs, α4β2* and α6β2* nAChRs on nigrostriatal dopamine terminals most likely play an important role. Data supporting this idea stems from experiments showing that the nicotine-mediated decline in LIDs is reduced or absent in animals with severe nigrostriatal damage (Bordia *et al.*, 2013; Huang *et al.*, 2011b; Quik *et al.*, 2013b; Quik *et al.*, 2012a). α4β2* nAChRs at other striatal sites, as well as in other brain regions, may also be involved since nicotine is still partially effective in severely lesioned rats (Quik *et al.*, 2013a). The localization of the CNS α7 nAChRs of relevance to the antidyskinetic effect of nicotine is currently not known (Quik *et al.*, 2013b). The idea that nAChRs throughout the brain modulate LID expression is not unlikely since the striatal dopaminergic system is functionally integrated with numerous other neurotransmitter systems.

6.1.4. Summary

Nicotine and/or nAChR agonists have been shown to reduce LIDs in experimental animal models. In addition, data from a small clinical trial show that oral nicotine decreases LIDs in PD patients. Nicotine and nAChR drugs also offer the benefit that they exhibit pro-cognitive and antidepressant properties, and have disease modifying potential (Dunbar *et al.*, 2011; Geerts, 2012; Lendvai *et al.*, 2013; Mineur and Picciotto, 2010; Philip *et al.*, 2010; Quik *et al.*, 2007b; Searles Nielsen *et al.*, 2012; Shimohama, 2009; Wirdefeldt *et al.*, 2011). These combined data provide a compelling rationale for the use nAChR drugs in the treatment of LIDs and other aspects of PD management.

6.2. Opioïd regulation

A role for enhanced peptidergic transmission, either opioidergic or not, has been proposed for the generation of LID on the basis of *in situ* hybridization studies showing that striatal peptidergic precursor expression consistently correlates with LID severity (Aubert *et al.*, 2007; Cenci *et al.*, 1998; Henry *et al.*, 2003; Tel *et al.*, 2002). Parkinsonian and dyskinetic states have been associated with different patterns of expression of precursors of the peptides. Parkinsonism is associated with increased expression of the opioid precursor proenkephalin (PENK) mRNA in striatal neurons projecting to the globus pallidus in rodents (GPe in primates) and a decreased prodynorphin (PDYN) mRNA expression in striatal neurons

projecting to the substantia nigra pars reticulata in rodents and primates and GPi in primates (Aubert *et al.*, 2007; Cenci *et al.*, 1998; Gerfen *et al.*, 1990; Henry *et al.*, 2003; Morissette *et al.*, 1999; Nisbet *et al.*, 1995; Quik *et al.*, 2002a; Tel *et al.*, 2002; Westin *et al.*, 2001). In the dyskinetic state, expression of PDYN mRNA is increased whereas PENK mRNA is unchanged versus controls, at least when the tissue was taken from animals killed at the peak of dyskinesia severity (Aubert *et al.*, 2007). Only few studies focused on the actual proteome and peptidome of both parkinsonian and dyskinetic states, three were conducted in the 6-OHDA-rodent model (Hanrieder *et al.*, 2011; Nilsson *et al.*, 2009; Valastro *et al.*, 2007) and two in the MPTP macaque model (Bourdenx *et al.*, 2014; Scholz *et al.*, 2008). These studies confirmed the results obtained from previous *in situ* hybridization-based studies. Moreover, the unbiased peptidomic approach lead to the identification of previous unreported peptides deriving from the classic precursors, some of them being specific of a given structure and/or DA-tone dependent (Bourdenx *et al.*, 2014; Klintenberg and Andren, 2005). However, the exact biological function of these new endogenous peptides remains to be determined.

The peptides processed from the different precursors bind with various affinities to the three classes of opioid peptide receptors, which have an overall inhibitory action (Hollt, 1986; Law *et al.*, 2000; Mansour *et al.*, 1994; Sadee *et al.*, 2005). Studies in rodents and macaques have shown an almost similar brain expression of the opioid peptide receptors in normal and pathological conditions (Aubert *et al.*, 2007; Johansson *et al.*, 2001; Mansour *et al.*, 1994). The total binding of opioid receptors decreases in the brain of DA-denervated animals and patients (Aubert *et al.*, 2007; Fernandez *et al.*, 1994; Johansson *et al.*, 2001) and further decreased in dyskinetic animals. In non human primates, Aubert and colleagues reported a reduction in μ and κ receptor binding in the GPi correlating with dyskinesia severity (Aubert *et al.*, 2007). It suggests that the more severe the LID are, the more profound is the decrease in total opioid receptor, κ, and μ binding in the GPi, as shown in rats (Johansson *et al.*, 2001) and in PD patients (Piccini *et al.*, 1997), reflecting an increased release of peptides.

On the clinical side, the non-subtype-selective opioid receptor antagonists naltrexone and naloxone have failed in clinic trials, showing almost no antidyskinetic effects (Fox *et al.*, 2004; Rascol *et al.*, 1994). However, μ-opioid receptor antagonists have been shown to efficiently reduce LID in non human primate models without affecting the antiparkinsonian action of L-DOPA (Henry *et al.*, 2001; Koprich *et al.*, 2011), thus suggesting that subtype-

selective agents would have a better clinical outcome. Taken together with the recent peptidomic-based results showing that regulation of peptidergic processing is highly structure-specific, this suggests that something more complex than a simple subtype-selective agent may be required to fully reverse the effects of the complex changes that occur in basal ganglia neuropeptide transmission in LID (Bourdenx *et al.*, 2014).

6.3. N/OFQ-NOP System

Nociceptin/orphanin FQ (henceforth N/OFQ) is a new member of the opioid family discovered in mid 90's by two separate groups of researchers who named it nociceptin (Meunier *et al.*, 1995) or orphanin FQ (Reinscheid *et al.*, 1995). N/OFQ is a heptadecapeptide with structural homologies with classical opioids, in particular dynorphin A, although the presence of a phenylalanine in its amino terminus instead of the "classical" tyrosine (as in opioid sequence) makes it unable to activate classical opioid receptors (μ, Δ and κ) with high affinity (Calo *et al.*, 2000b; Mogil and Pasternak, 2001). Indeed, N/OFQ is the endogenous ligand of the so-called Opioid Receptor Like 1 (ORL 1) receptor, recently renamed N/OFQ peptide (NOP) receptor, which was cloned one year before the isolation of N/OFQ (Mollereau *et al.*, 1994) and crystallized very recently (Thompson *et al.*, 2012). The NOP receptor is a classical GPCR which couples to Gi/o, leading to inhibition of cAMP accumulation, closing of voltage gated Ca2+ channels, and opening of inwardly rectifier K+ channels. These effects result in generally inhibitory actions over neuronal firing and neurosecretion. In addition, N/OFQ can activate mitogen-activated protein (MAP) kinases, among which ERK (New and Wong, 2002).

In keeping with the widespread distribution of N/OFQ and its receptor in the brain and spinal cord, N/OFQ regulates a number of central functions such as pain perception, mood, reward, food intake and locomotion (Chiou *et al.*, 2007; Mogil and Pasternak, 2001). In addition, it regulates the cardiovascular and respiratory systems, the gastrointestinal and the urogenital tracts, and the immune system, offering diverse therapeutic opportunities to NOP receptor ligands, ranging from cough and overactive bladder treatment, to pain and drug abuse (Lambert, 2008).

Relevant to PD, the N/OFQ-NOP receptor system is highly expressed in the basal ganglia (Anton *et al.*, 1996; Neal *et al.*, 1999a; Neal *et al.*, 1999b). High levels of N/OFQ+ neurons and fibers were detected with in situ hybridization and immunohistochemistry in the globus pallidus, entopeduncular nucleus, and substantia nigra in both SNc and SNr, while in striatum only few, scattered N/OFQ+ neurons were evident. The distribution of the NOP receptor substantially matches that of N/OFQ, with the exception of the subthalamic nucleus where high levels of NOP but only scattered N/OFQ+ neurons were found. Significant levels of N/OFQ immunoreactivity (Witta *et al.*, 2004) and expression (Peluso *et al.*, 1998), as well as N/OFQ binding and NOP receptor expression (Berthele *et al.*, 2003) were also measured in the human basal ganglia. A notable difference between the human and rodent brain, are the high levels of NOP receptor/N/OFQ binding in the caudate/putamen, also observed in non human primates (Bridge *et al.*, 2003).

The NOP receptor is expressed in midbrain dopaminergic neurons of the ventral tegmental area and SNc (Norton *et al.*, 2002), and sorted both to the somatodendritic and nerve terminal compartments. NOP receptor activation inhibits the firing of nigral DA neurons (Marti *et al.*, 2004b) and striatal presynaptic DA release (Flau *et al.*, 2002), an effect correlated with motor inhibition (Marti *et al.*, 2004b).

The NOP receptor is a druggable receptor which has unique pharmacological properties with respect to the classical opioid systems, first of all the insensitivity to naloxone (Calo *et al.*, 2000b). This peculiarity has contributed to the definition of NOP as a non-opioid member of the opioid receptor family. Since the initial structure-activity relation studies on N/OFQ analogues (Guerrini *et al.*, 1997; Reinscheid *et al.*, 1996), several NOP selective ligands have been developed by academic and industrial groups (for reviews see (Calo *et al.*, 2000a; Zaveri *et al.*, 2005). Potent and NOP selective peptidic antagonists, e.g. UFP-101 (Calo *et al.*, 2005), partial agonists, e.g. Dooley's peptides or ZP120 (Rizzi *et al.*, 2002), and full agonists, e.g. UFP-112 (Calo *et al.*, 2011), are now available. Small molecules NOP receptor antagonists have also been synthesized and characterized, such as J-113397 (Ozaki *et al.*, 2000), and the more potent and selective SB-612111 (Zaratin *et al.*, 2004) and Compound 24 (Goto *et al.*, 2006). Conversely, small molecules NOP agonists presently available, such a Ro 65-6570 (Rover *et al.*, 2000), Ro 64-6198 (Jenck *et al.*, 2000) or SCH221510 (Varty *et al.*, 2008) do not possess optimal selectivity for the NOP over the classical opioid receptors.

The first evidence linking N/OFQ with PD was provided in 2004 (Marti *et al.*, 2004a), showing that a selective NOP receptor antagonist injected into SNr was able to reverse haloperidol-induced catalepsy, simultaneously normalizing the associated rise in glutamate levels in rats. This observation was confirmed one year later through systemic administration of the small molecule NOP antagonist J-113397 (Marti *et al.*, 2005). In this seminal paper, it was also reported that systemic J-113397 was able to attenuate motor deficit in 6-OHDA-hemilesioned rats, mimicking the effect of L-Dopa. It was also established that NOP antagonists exert their antiparkinsonian effects acting into the SNr, likely because in this area extracellular N/OFQ levels rise as a consequence of DA neuron degeneration (Marti *et al.*, 2005) or functional impairment of DA transmission (Marti *et al.*, 2010). In 6-OHDA hemilesioned rats, elevation of N/OFQ levels correlates with an increase in N/OFQ expression in SNr neurons (Marti *et al.*, 2005; Marti *et al.*, 2010; Norton *et al.*, 2002), which was also found in MPTP-treated mice (Di Benedetto *et al.*, 2009; Gouty *et al.*, 2010). In view of the motor inhibiting effects of exogenous and endogenous N/OFQ in SNr (Marti *et al.*, 2004b; Marti *et al.*, 2009), the elevation of nigral N/OFQ levels is likely to contribute to parkinsonian-like motor deficit. Interestingly, a similar elevation of N/OFQ levels were found in the CSF of PD patients, possibly indicating a pathogenic response of N/OFQ to DA neuron loss also in humans (Marti *et al.*, 2010). The beneficial effect of J-113397 and UFP-101 in 6-OHDA hemilesioned rats was further confirmed using different NOP receptor antagonists (Marti *et al.*, 2013; Marti *et al.*, 2008; Volta *et al.*, 2010; Volta *et al.*, 2011), alone or in combination with L-Dopa (Marti *et al.*, 2008; Marti *et al.*, 2007).

Mechanistic studies using microdialysis combined to behavioral testing revealed that nigral endogenous N/OFQ modulates nigral GABA output neurons and movement initiation. In fact, pharmacological blockade of the NOP receptor into SNr reduced nigral glutamate and increased nigral GABA levels, leading to inhibition of GABA neurons projecting to the ventro-medial thalamus, and relief from akinesia (Marti *et al.*, 2008; Marti *et al.*, 2007; Volta *et al.*, 2011). Direct evidence that this action involves thalamo-cortical projections and the processing of motor information in primary motor cortex (M1) was provided with intracortical microstimulation (ICMS) technique, by showing an increase in M1 neurons excitability in rats injected with NOP antagonists into SNr (Marti *et al.*, 2009).

In 2008, it was proven that J-113397 alone was effective in reducing motor deficits in MPTP-treated mice and macaques (Viaro *et al.*, 2008), and that it potentiated the antiparkinsonian

effect of L-Dopa in MPTP-treated marmosets (Visanji *et al.*, 2008). Although both supportive of a potential use of NOP antagonists in humans, these studies in non human primates revealed that the effect of J-11397 was dose-dependent with reversal of action at high doses (Viaro *et al.*, 2008), and that J-113397 enhanced the effect of L-Dopa at the cost of causing the appearance of dyskinesia (Visanji *et al.*, 2008).

The question of whether NOP receptor antagonists are dyskinesiogenic was directly addressed in a recent paper showing that acute systemic administration of J-113397 worsened the severity of AIMs in dyskinetic rats (Marti *et al.*, 2012). This effect was replicated by i.c.v. or intranigral injection of UFP-101, consistent with the view that NOP antagonists act where N/OFQ tone is elevated. In fact, injection of UFP-101 in striatum, an area where N/OFQ tone is low or absent, and NOP receptor up-regulated after DA denervation, was without effect (Marti *et al.*, 2012). Although in rats the prodyskinetic effect was mild and limited to the limb subtype of AIMs, these data substantially confirmed previous findings in marmosets (Visanji *et al.*, 2008) warning of the potential motor side effects of overdosing NOP antagonists as an adjunct to L-Dopa therapy.

The finding that NOP receptor blockade worsens AIM expression, suggested that endogenous N/OFQ might physiologically oppose LID. In fact, acute icv injection of N/OFQ or systemic administration of Ro 65-6570 (NOP agonist) mitigated LID expression in rats, being equally effective against axial, limb and orolingual AIMs (Marti *et al.*, 2012). The antidyskinetic effect was observed at doses that *per se* did not cause hypolocomotion, a typical effect of NOP receptor agonists (Devine *et al.*, 1996; Jenck *et al.*, 1997; Marti *et al.*, 2004a; Marti *et al.*, 2009; Reinscheid *et al.*, 1995), possibly indicating a specific interference with dyskinesia pathways. Opposite to NOP antagonists, N/OFQ attenuated dyskinesia more potently when injected in striatum than SNr, an area characterized by low N/OFQ tone and up-regulated NOP receptors.

In vivo microdialysis revealed that N/OFQ prevented LID expression through an action upon striatal GABAergic MSNs projecting to SNr. In fact, i.c.v. N/OFQ markedly attenuated the rise of SNr GABA release associated with L-Dopa induced AIM expression (Mela *et al.*, 2007), a neurochemical response associated with striatal D1 receptor activation (Mela *et al.*, 2012). Consistently, N/OFQ also prevented the reduction of GABA release in ventro-medial thalamus associated with LID, an index of overinhibition of the nigral output (Marti *et al.*,

2012). As previously reviewed, up-regulation of striatal D1 signalling in LID is associated with an increased activity along the Ras/MEK/ERK kinase pathway (Feyder *et al.*, 2011; Valjent *et al.*, 2005), and a loss of neuron capability to depotentiate striatal synaptic response after LTP induction (Picconi *et al.*, 2003). Consistent with an inhibitory action of N/OFQ upon striatal D1 signalling (Olianas *et al.*, 2008), application of N/OFQ to striatal slices of naïve animals prevented the increase in ERK phosphorylation induced by a D1 agonist, and fully restored the depotentiation in slices treated with a D1 agonist (Marti *et al.*, 2012). The potential of NOP agonists as antidyskinetic was further confirmed in MPTP-treated macaques, where the small molecule Ro 65-6570 was able to attenuate dyskinesia without compromising the antiparkinsonian effect of L-Dopa (Marti *et al.*, 2012). Although the effect was overall mild (30%) and significant for the dystonic but not the choreiform component (here a trend for a reduction was clear, though), these data provide a solid background for testing more selective NOP agonists for their ability to acutely rescue motor function under LID.

In conclusion, N/OFQ appears to play a pathogenic role in PD. In particular, elevation of N/OFQ transmission in SNr following DA neuron loss might exacerbate the physiological, inhibitory role of N/OFQ over movement, justifying the use of NOP receptor antagonists as symptomatic antiparkinsonian drugs. Opposite to SNr, DA neuron loss is associated with reduction of N/OFQ expression and up-regulation of NOP receptors in striatum, which might also contribute to dysregulation of D1 transmission in striato-nigral MSNs. In this case, NOP agonists, by restoring an inhibitory control over D1 signalling, might work to oppose LID expression. Studies with more selective NOP agonists are needed to confirm these data, and prove that N/OFQ also prevents the development of sensitization to L-Dopa, which underlies LID.

Considering the motor side effects associated with overdosing NOP antagonists in SNr (exacerbation of LID) and NOP agonists in striatum (hypolocomotion), a fascinating possibility to provide a balanced action in these two areas using a NOP partial agonist can be put forward. Indeed, such a drug would be expected to act as an antagonist under conditions of high extracellular levels of endogenous N/OFQ (i.e. in SNr) and as an agonist where endogenous N/OFQ tone is low or absent, and NOP receptors are up-regulated (i.e. in striatum), thus providing a combined antiparkinsonian and antidyskinetic effect.

6.4. Additional nuclei involvement in LID pathophysiology

As the main target of the nigral DA neurons, the striatum, and generally the other basal ganglia sub nuclei (i.e. GPe, STN, GPi, SNr), have received most attention to understand the pathophysiology of LID.
However, little remains known of the adaptations occurring in other structures following a chronic L-Dopa treatment.

First, a functional study revealed that resonant cortical oscillations are associated with LID (Halje *et al.*, 2012). Interestingly, the authors showed a direct link between the cortical oscillations and the DA D1 receptor. Local delivery of a D1 receptor antagonist (SCH23390) at the surface of the primary motor cortex decreased both cortical oscillations and LID in dyskinetic 6-OHDA-lesioned rats compared to vehicle-treated control animals, suggesting a key role of cortical oscillations in the generation of AIMs.

Then, 2-deoxyglucos (2-DG) studies showed modification in 2-DG accumulation in structures outside of the basal ganglia both in PD and LID. Interestingly, Mitchell et al. showed that, besides the classic 2-DG uptake pattern in the basal ganglia (Bezard *et al.*, 2001c; Gnanalingham *et al.*, 1995), the Lateral Habenula (LHb) and the Pedunculopontine Tegmental nucleus (PTg) stood up among several structures as strongly affected non-basal ganglia nuclei, showing dramatic increase in 2-DG accumulation in parkinsonism (Mitchell *et al.*, 1992; Mitchell *et al.*, 1989). Recently, Guigoni and co-workers shown a decreased in 2-DG uptake in the bed nucleus of the stria terminalis (BST) only in L-Dopa dyskinetic MPTP-treated macaques (Guigoni *et al.*, 2005c). In addition, neuronal activity of the locus coeruleus, containing the largest population of central noradrenergic neurons, is altered in dyskinetic 6-OHDA-lesioned rats following *in vivo* single-unit extracellular recordings (Miguelez *et al.*, 2011). Finally, recent studies demonstrated that the prefrontal cortex, the hippocampus and the amygdala displayed a modified monoaminergic neurochemistry both in dyskinetic 6-OHDA-lesioned rats and MPTP-treated macaques following a chronic L-Dopa treatment (Engeln *et al.*, 2014; Navailles *et al.*, 2011a). Taken together, these studies suggest that structures outside of the basal ganglia nuclei could be involved in LID pathophysiology

Consequently, the above-mentioned data further support the need to evaluate the functional involvement of regions outside of the basal ganglia to fully uncover the pathophysiological mechanisms underlying LID.

As mentioned in the foreword, decipher the roles of these additional nuclei in the pathogenesis of LID will be the focus of my PhD.

Results

1. Publication 1: Immediate-early genes expression in structures outside the basal ganglia is associated to L-Dopa-induced dyskinesia

Matthieu F Bastide, Sandra Dovero, Giselle Charron, Gregory Porras, Christian E Gross, Pierre-Olivier Fernagut and Erwan Bézard
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As underlined by the introductory review, basal ganglia motor circuits have received most attention to understand the pathophysiology of LID, both in fundamental and clinical research. However, the myriad of dopaminoceptive structures, outside of the basal ganglia, that are likely to be affected by the exogenously produced dopamine have received little, if any, attention although they might play a key role in mediating LID. Therefore, in order to identify structures outside of the basal ganglia potentially affected by a chronic L-Dopa treatment, we used an unbiased stereological approach to achieve a whole brain screening of dyskinetic 6-OHDA-lesioned rats with 4 IEGs: ΔFosB, ARC, Zif268 and FRA2 compared to non-dyskinetic 6-OHDA-lesioned rats. IEGs are a class of genes rapidly transcribed in response to an external stimulus allowing us to identify brain nuclei displaying a transcriptional response specifically related to LID. Such approach notably shed light upon 9 structures located outside of the basal ganglia and displaying an overexpression of at least 3 IEGs. Among the identified nuclei, the oval and juxta nuclei of the bed nucleus of the stria terminalis, the lateral habenula, the pontine nuclei and the cuneiform nucleus demonstrate a significant correlation between at least one IEG expression profile and LID severity. In this study, we therefore identified non-motor domains of cortico-sub-cortical loops that could be involved in LID pathophysiology.

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Immediate-early gene expression in structures outside the basal ganglia is associated to L-DOPA-induced dyskinesia

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ABSTRACT

Long-term L-3,4-dihydroxyphenylalanine (L-DOPA) treatment in Parkinson's disease (PD) leads to L-DOPA-induced dyskinesia (LID), a condition thought to primarily involve the dopamine D1 receptor-expressing striatal medium spiny neurons. Activation of the D1 receptor results in increased expression of several molecular markers, in particular the members of the immediate-early gene (IEG) family, a class of genes rapidly transcribed in response to an external stimulus. However, several dopaminoceptive structures in the brain that are likely to be affected by the exogenously produced DA have received little attention although they might play a key role in mediating those L-DOPA-induced abnormal behaviours. ΔFosB, ARC, FRA2 and Zif268 IEGs expression patterns were thus characterised, using unbiased stereological methods, in the whole brain of dyskinetic and non-dyskinetic rats to identify brain nuclei displaying a transcriptional response specifically related to LID. Within the basal ganglia, the striatum and the substantia nigra pars reticulata showed an increased expression of all four IEGs in dyskinetic compared to non-dyskinetic rats. Outside the basal ganglia, there was a striking increased expression of the four IEGs in the motor cortex, the bed nucleus of the stria terminalis, the dorsal hippocampus, the pontine nuclei, the cuneiform nucleus and the pedunculopontine nuclei. Moreover, the zona incerta and the lateral habenula displayed an overexpression of ΔFosB, ARC and Zif268. Among these structures, the IEG expression in the striatum, the bed nucleus of the stria terminalis, the lateral habenula, the pontine nuclei and the cuneiform nucleus correlate with LID severity. These results illustrate a global transcriptional response to a dyskinetic state in the whole brain suggesting the possible involvement of these structures in LID.

Introduction

The most effective symptomatic therapy in Parkinson's disease (PD) remains the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA). Long-term treatment leads to involuntary aimless movements called L-DOPA-induced dyskinesia (LID) (Fahn, 2008; Stocchi et al., 1997). Loss of dopamine in PD induces complex modifications in cellular signalling with numerous pathways showing altered responses to dopaminergic stimulation in the dopamine-depleted striatum (Bezard et al., 2001; Jenner, 2008). Chronic L-DOPA treatment further enhances the signalling alterations. The striatal dopamine D1 receptor (D1R) signalling pathways have consistently been shown to be critically involved in LID genesis and manifestation (Bezard et al., 2001; Jenner, 2008). D1R stimulation results in increased expression of several molecular markers (Feyder et al., 2011), in particular the members of the immediate-early gene (IEG) family, a class of genes rapidly transcribed in response to an external stimulus (Okuno, 2011). The ΔFosB, activity-regulated cytoskeleton-associated protein (ARC) (also known as Arg3.1), FRA2 and Zif268 IEGs (Granado et al., 2008; Westin et al., 2007; Wirtshafter, 2007) show a concomitant increased expression in the striatum of dyskinetic rats with different expression patterns (Cenci et al., 1999; Ebihara et al., 2011; Sgambato-Faure et al., 2005).

As the main target of the nigral DA neurons, the striatum has received much attention in regard to understanding the pathophysiology of LID. However, the myriads of dopaminoceptive structures in the brain that are likely to be affected by the exogenously produced dopamine have received little, if any, attention although they might play a key role in mediating those L-DOPA-induced abnormal behaviours. Therefore, we here characterised ΔFosB, ARC, FRA2 and Zif268 expressions in the whole brain of dyskinetic and non-dyskinetic rats to identify the brain nuclei displaying a transcriptional response specifically related to LID.

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Material and methods

Experimental protocol

Adult Sprague–Dawley male rats (Charles River Laboratories, Lyon, France), weighing 175–200 g at the beginning of the experiment, were used. They were housed under standard laboratory condition in a 12-hour light/12-hour dark cycle with free access to food and water. The experimental protocol was approved by the Ethical Committee of the Bordeaux Segalen University CE50 under licence no. 5012099-A.

On day 0, unilateral injection of 6-hydroxydopamine (2.5 μl at 3 μg/μl) was performed in the right medial forebrain bundle (AP = −3.7 mm; ML = +1.6 mm; DV = −8 mm relative to Bregma) in rats treated 30 min before with citalopram at 1 mg/kg i.p. (an inhibitor of serotonin re-uptake) and with desipramine hydrochloride at 20 mg/kg i.p. (an inhibitor of noradrenalin re-uptake) according to previously published procedures (Berthet et al., 2009; Porras et al., 2012; Schuster et al., 2008). Only the animals displaying both an impaired stepping test (Olsson et al., 1995; Pioli et al., 2008) assessed on days 18 to 20 and a loss of tyrosine hydroxylase-immunopositive fibres in the striatum greater than 95% (Bezard et al., 2001; Jenner, 2008) were retained for final analysis (Fig. 1M).

From day 21 onwards, rats received once daily an i.p. injection of a combined dose of benserazide (15 mg/kg, i.p.) and L-DOPA (3 mg/kg, i.p.) for 18 days. This L-DOPA dose is similar to the EC50 value (12.5 mg/kg of benserazide and 3.2 mg/kg of L-DOPA) required to allow a gradual development of dyskinesia (Putterman et al., 2007). On day 39, 1/3 of the rats were found non-dyskinetic while 2/3 were found dyskinetic, after being rated by a trained investigator as previously described (Berthet et al., 2012; Meissner et al., 2006; Porras et al., 2012; Schuster et al., 2008, 2009). The 4 abnomal involuntary movements (AIMs) categories (limb, axial, orolingual, and locomotive) were scored using a validated rating scale (Cenci et al., 1998; Lundblad et al., 2002) for 1 min every 20 min for 2 h (total 4 observations; maximal score for each observation, 16; maximal total score per session, 64). Since the study aimed at defining the transcriptional response induced by chronic L-DOPA treatment, non-dyskinetic lesioned rats (n = 5) were taken as the reference experimental group of dyskinetic lesioned rats (n = 5).

Tissue preparation

On day 40, 1 h after the last L-DOPA injection, i.e. at the peak of behavioural effect, rats were deeply anesthetised with chloral hydrate (400 mg/kg, i.p., VWR) and perfused transcardially with 0.9% NaCl followed by ice-cold 4% formaldehyde in 0.1 M sodium phosphate buffer (PBS). Brains were removed, postfixed overnight in the same fixative (4 °C), then cryoprotected for 48 h at 4 °C in 20% sucrose (diluted in PBS). Brains were frozen in isopentane at −45 °C and stored at −80 °C until sectioning. 50 μm-thick cryostat-cut coronal sections were collected in PBS containing 0.2% sodium azide and stored at 4 °C pending immunohistochemistry.

Immunohistochemistry

After three washes in PBS, free-floating sections were incubated for 10 min in 3% H_2O_2 (Sigma-Aldrich) at room temperature (RT) to quench endogenous peroxidase. Sections were then transferred for 30 min at RT in a blocking solution containing 1/50 bovine serum albumine (BSA) and 0.3% Triton X-100 (Sigma-Aldrich) in PBS. Sections were incubated for 12 h at RT with rabbit polyclonal anti-FosB/ΔFosB (sc-48), anti-ARC (sc-15325), anti-Zif268 (sc-189), anti-FRA2 antibody (sc-604) (Santa Cruz Biotechnology), all diluted at 1:500 or mouse monoclonal anti-tyrosine hydroxylase (MAB318, Millipore) diluted at 1:10,000 in PBS containing 1/500 BSA and 0.3% Triton X-100. After three washes in PBS (10 min each), sections were incubated for 30 min at RT with anti-rabbit or anti-mouse labelled Polymer-HRP (Dako). After thorough washing, the staining was revealed with a DAB peroxidase substrate kit (Vector). The specificity of the immunostaining was assessed by omission of the primary or the secondary antibody. After processing, tissue sections were mounted onto gelatin-coated slides, air-dried, dehydrated and coverslipped with Eukitt mounting medium (Sigma-Aldrich) for light microscopic inspection.

Data analysis

The number of IEG-immunopositive neurons was obtained applying the optical fractionator (Engeln et al., 2012; Pioli et al., 2008; West et al., 2004) unbiased stereological method using a Leica DM6000B microscope with Mercator Pro software (ExploraNova, version 7.9.8). Immuno-labelled cells were counted by a blind investigator on every 6th section, a sampling adapted to the studied brain nuclei. For each section, the boundaries of the regions were first delineated at low magnification (×2.5) and counting was performed at high magnification (×40). Stereological details for each analysed brain nucleus are presented in Table 1. Mean ± standard deviation (S.D.) of these values was calculated for each group (5 rats per group). The stereological error coefficients range between 3% (e.g. striatum) and 17% (e.g. rostral zona incerta) depending on the size and thickness of the structure in accordance with the literature e.g. (Gundersen and Jensen, 1987; Schmitz and Hof, 2000; Slomianka and West, 2005; West et al., 1991).

Statistical analysis

Statistical analyses were performed using a two-way ANOVA. If significant, ANOVAs were followed by post hoc t tests corrected for multiple comparisons by the method of Bonferroni. All data were normally distributed, and significance levels of *t* test comparisons were adjusted for inequality of variances when appropriate. The provided F values correspond to the interaction between independent variables: Condition (dyskinetic or non-dyskinetic) × side (lesioned or unlesioned). Correlations between LID and cell counts were performed using Spearman correlation.

Results

The basal ganglia display an overexpression of IEGs in LID

Within the cortico-basal ganglia motor loops, as expected, ΔFosB, ARC, FRA2 and Zif268 IEGs are significantly overexpressed in the dorsolateral, ventrolateral, dorsomedial and ventromedial part of the lesioned striatum of dyskinetic rats compared to non-dyskinetic rats (Figs. 1CF, 2CF, 3CF and 4CF). Likewise, in the motor cortex (M1) and the substantia nigra pars reticulata (SNr), the 4 IEGs displayed a significant increased expression between the lesioned side of dyskinetic and non-dyskinetic rats (Figs. 1IL, 2IL, 3IL and 4IL). However, the other structures of the basal ganglia, the subthalamic nucleus and the external globus pallidus (GPe) displayed no staining.

LID increase IEGs expression outside the basal ganglia

In the limbic system, 3 brain nuclei of the bed nucleus of the stria terminalis (BST): the oval (oBST), juxta capsular (jBST) and medial (mBST) nuclei exhibited a significant increased expression of the 4 IEGs on the lesioned side of dyskinetic rats compared to non-dyskinetic (Figs. 5CF, 6CF, 7CF and 8CF). A similar expression pattern was also found in the hippocampus where the IEGs were significantly overexpressed in CA1, CA3 and in the dentate gyrus (DG) (Figs. 5U, 6U, 7U and 8U).

At the crossroad between the limbic system, the basal ganglia and the dopamine/serotonine pathways, the lateral part of the habenula (LHb) displayed a significant overexpression of ΔFosB, ARC and Zif268

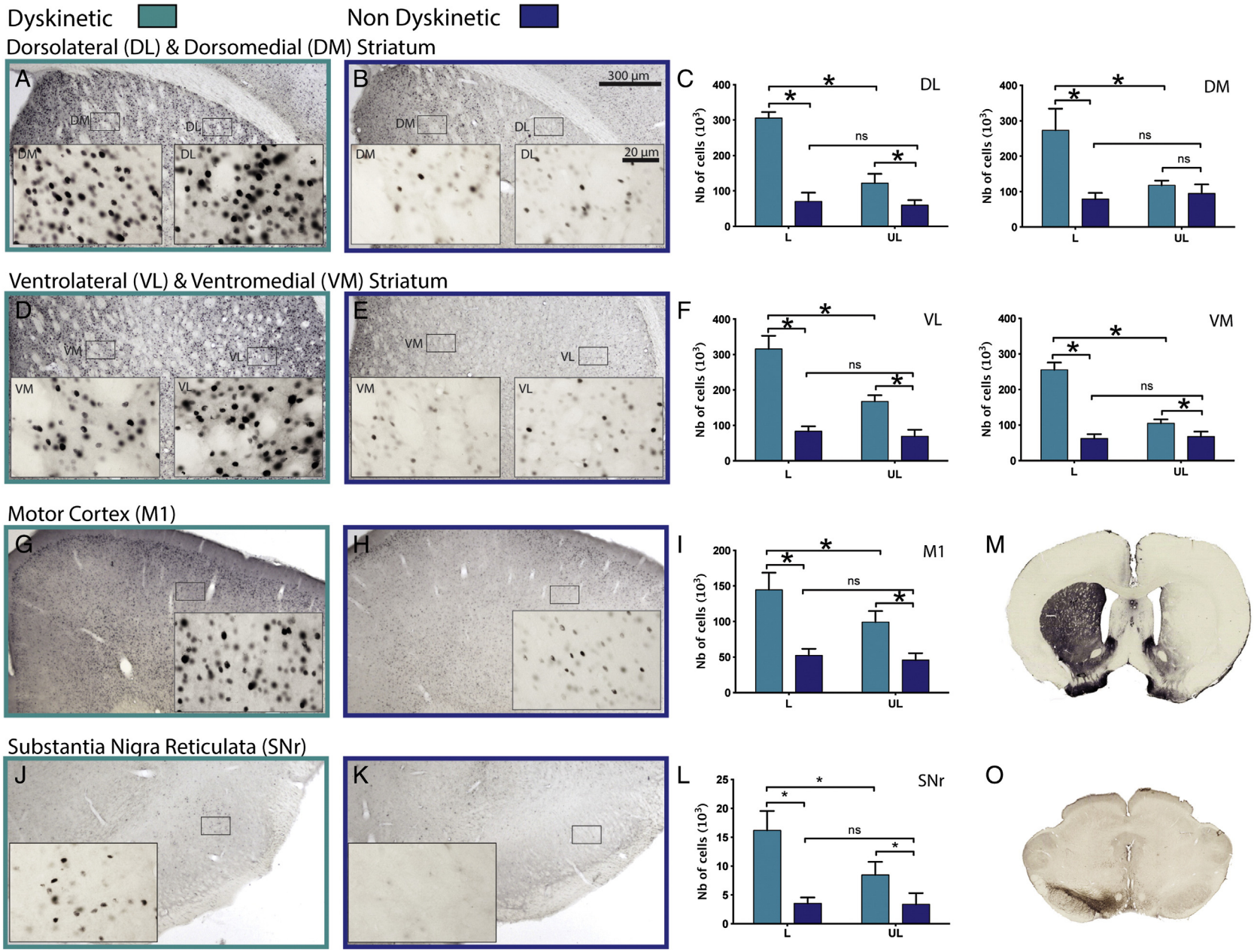


Fig. 1. Stereological counting of ΔFosB immuno-positive cells in the cortico-basal ganglia motor loop in dyskinetic (light blue) and non-dyskinetic (dark blue) 6-OHDA-lesioned rats. Representative examples of staining, scale bar 300 µm (with an inset magnification, scale bar 20 µm), are shown on the left side while quantitative results are displayed on the right side (shown as mean ± SD; *$p < 0.05$). A–C dorsolateral (DL) $F_{[1,16]} = 84.27$, $p < 0.001$ and dorsomedian (DM) $F_{[1,16]} = 29.9$, $p < 0.001$ striatum; D–F ventrolateral (VL) $F_{[1,16]} = 40.46$, $p < 0.001$ and ventromedian (VM) $F_{[1,16]} = 138$ $p < 0.001$ striatum; G–I, M1 motor cortex $F_{[1,16]} = 7.527$, $p < 0.05$; J–L, substantia nigra pars reticulata (SNr) $F_{[1,16]} = 13.31$, $p < 0.01$. M, Representative example of tyrosine hydroxylase immunostaining in the striatum of L-DOPA-treated unilateral 6-OHDA lesioned rats. O, Representative example of tyrosine hydroxylase immunostaining in the SNc of L-DOPA-treated unilateral 6-OHDA lesioned rats.

Table 1
Stereological counting parameters. STR = striatum, M1 = motor cortex, mBST oBST jBST = medial, oval and juxta part of the bed nucleus of the stria terminalis, lHb and mHb = lateral and medial part of the habenula, DG = dentate gyrus, rZI = rostral zona incerta, SNr = substantia nigra reticulata, Pn = pontine nuclei, PTg = pedunculopontine tegmental nucleus, CnF = cuneiform nucleus.

	STR	M1	mBST	oBST	jBST	lHb	mHb	CA1	CA2	CA3	DG	rZI	SNr	Pn	PTg	CnF
Counting frames (μm)	60 × 80	80 × 80	60 × 60	60 × 60	60 × 60	60 × 60	60 × 60	80 × 40	80 × 40	80 × 40	80 × 40	80 × 40	80 × 60	80 × 60	60 × 60	60 × 60
Spacing (μm)	200 × 250	300 × 300	150 × 150	80 × 80	80 × 80	100 × 100	80 × 80	200 × 50	100 × 50	200 × 50	200 × 50	200 × 50	240 × 180	250 × 200	150 × 150	150 × 150
Number of sections	10	10	1	1	1	3	3	4	4	4	4	3	6	3	2	2

while only ARC expression was increased in the medial part (MHb) (Figs. 5L, 6L and 8L). The same expression pattern was found in the rostral part of the zona incerta (rZI) where 3 IEGs were significantly overexpressed (Figs. 5I, 6I, and 8I).

In the brainstem, the number of ΔFosB, ARC, FRA2 and Zif268 immuno-positive cells was significantly greater in the pontine (Pn) and cuneiform nuclei (CnF) (Figs 5OR, 6OR, 7OR and 8OR) whereas the pedunculopontine tegmental nucleus (PTg) only displayed an overexpression of ΔFosB, ARC and Zif268 (Figs. 5R, 6R and 8R) on the lesioned side of dyskinetic rats compared to non-dyskinetic.

IEG expression is altered on the unlesioned side of dyskinetic rats

In dyskinetic rats, the 4 IEGs showed an overexpression in all the structures mentioned above on the lesioned side compared to the unlesioned side. However, interestingly, IEGs displayed an increased expression on the unlesioned side of dyskinetic rats compared to non-dyskinetic rats, both in the basal ganglia and in some structures outside.

In the basal ganglia, the number of ARC and FRA2 immuno-positive cells was significantly greater in the 4 parts of the striatum while ΔFosB was overexpressed only in the dorsolateral, ventrolateral and ventromedial parts (Figs. 1CF, 2CF and 3CF). SNr and M1 also showed a significant increased expression of ΔFosB and Zif268 whereas ARC was overexpressed only in the SNr (Figs. 1IL, 2IL and 4IL).

Outside the basal ganglia, oBST, mBST and LHb displayed a significant overexpression of ΔFosB and ARC (Figs. 5CFL and 6CFL) while rZI showed an increased expression only for ARC (Fig. 6I). In the hippocampus, the number of ARC immune-positive cells was significantly greater in CA1, CA3 and DG (Fig. 6U). Zif268 and FRA2 were overexpressed only in CA3 (Figs. 7U and 8U) and ΔFosB in DG (Fig. 5U). In the brainstem, only ARC displayed an increased expression in the CnF (Fig. 6R).

Discussion

L-DOPA, the gold standard treatment for PD, rapidly induces fluctuations and LID, the latter being so far associated with both presynaptic and postsynaptic mechanisms at the striatal level (Bezard et al., 2001; Jenner, 2008). The present study, building upon scarce but intriguing evidences in the literature (e.g. (Guigoni et al., 2005b; Halje et al., 2012; Miguelez et al., 2011)), systematically assessed the dyskinesia-related increases in expression of 4 IEGs: ΔFosB, ARC, FRA2 and Zif268 in the whole brain of a rat model of PD. While the striatum is undoubtedly central in LID pathophysiology as local infusions in the striatum elicit LID (Buck and Ferger, 2008; Carta et al., 2006), the present study unravels stunning correlations between IEG expression and LID severity in brain nuclei outside the basal ganglia, such as the BST, lateral habenula, pontine nuclei and cuneiform nucleus.

The study is however not without limitations. L-DOPA dose was carefully selected to be just enough for inducing dyskinesia in the majority of animals while still allowing some to have a score of 0 (Putterman et al., 2007). Most studies actually compare highly dyskinetic to low dyskinetic 6-OHDA-lesioned rats (Fiorentini et al., 2006; Rangel-Barajas et al., 2011) while the non-dyskinetic animals here displayed a score of 0. As the extent of the lesion is an obvious factor for susceptibility to develop LID (Guigoni et al., 2005a), we selected animals with the exact same extent and pattern of nigrostriatal denervation (Fig. 1M) (Berthet et al., 2012; Porras et al., 2012; Schuster et al., 2008). Finally, while it would be tempting to relate the changes in IEG expression to changes in electrophysiological activity of the considered neuronal structures, one should bear in mind that such a relationship, although generally assumed, has not been demonstrated for most IEGs (Loebrich and Nedivi, 2009) and in particular for those studied here. Therefore, increased expression of an IEG should be seen as an increased transcriptional activity and not taken as an increase in electrophysiological activity that remains to be demonstrated.

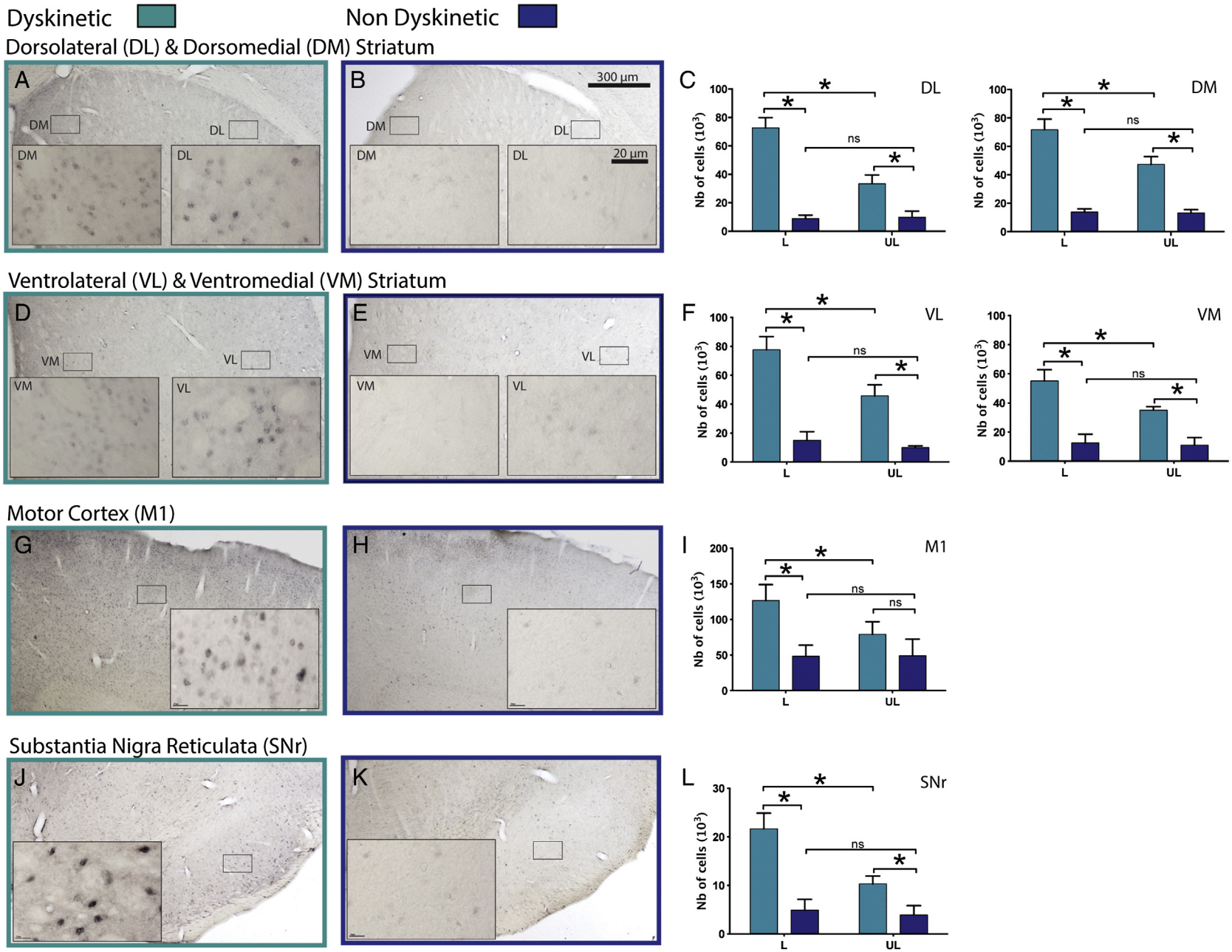


Fig. 2. Stereological counting of ARC immuno-positive cells in the cortico-basal ganglia motor loop in dyskinetic (light blue) and non-dyskinetic (dark blue) 6-OHDA-lesioned rats. Representative examples of staining, scale bar 300 µm (with an inset magnification, scale bar 20 µm), are shown on the left side while quantitative results are displayed on the right side (shown as mean ± SD; $^{*}p < 0.05$). A–C dorsolateral (DL) $F_{[1.16]} = 71.03$, $p < 0.001$ and dorsomedian (DM) $F_{[1.16]} = 29.71$, $p < 0.001$ striatum; D–F ventrolateral (VL) $F_{[1.16]} = 19.84$, $p < 0.001$ and ventromedian (VM) $F_{[1.16]} = 13.54$, $p < 0.001$ striatum; G–I, M1 motor cortex $F_{[1.16]} = 7.344$, $p < 0.05$; J–L, substantia nigra pars reticulata (SNr) $F_{[1.16]} = 24.74$, $p < 0.001$.

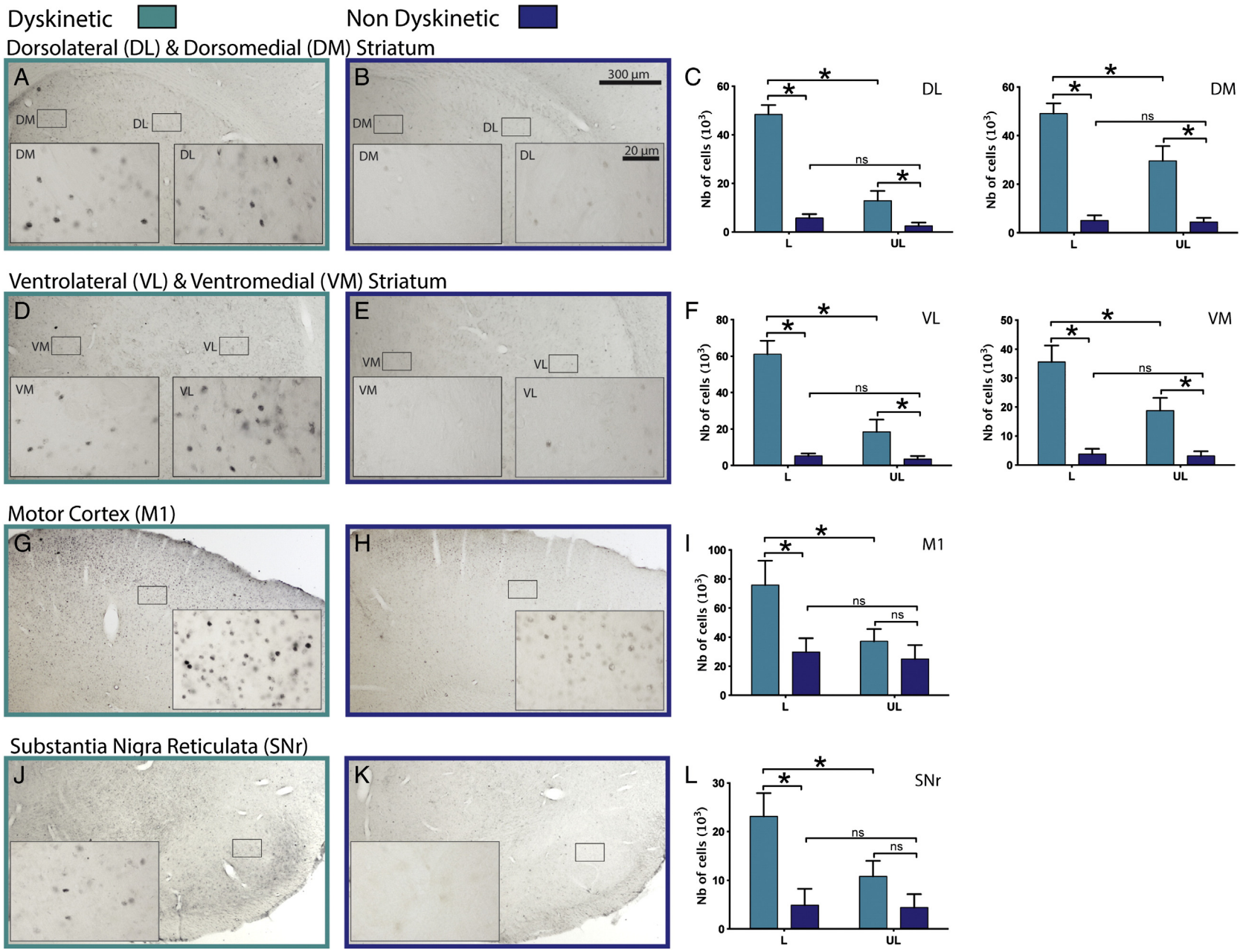


Fig. 3. Stereological counting of FRA2 immuno-positive cells in the cortico-basal ganglia motor loop in dyskinetic (light blue) and non-dyskinetic (dark blue) 6-OHDA-lesioned rats. Representative examples of staining, scale bar 300 µm (with an inset magnification, scale bar 20 µm), are shown on the left side while quantitative results are displayed on the right side (shown as mean ± SD; *$p < 0.05$). A–C dorsolateral (DL) $F_{[1.16]} = 152.1$, $p < 0.001$ and dorsomedian (DM) $F_{[1.16]} = 28.72$, $p < 0.001$ striatum; D–F ventrolateral (VL) $F_{[1.16]} = 80.18$, $p < 0.001$ and ventromedian (VM) $F_{[1.16]} = 23.30$, $p < 0.001$ striatum; G–I, M1 motor cortex $F_{[1.16]} = 10.83$, $p < 0.01$; J–L, substantia nigra pars reticulata (SNr) $F_{[1.16]} = 13.47$, $p < 0.01$.

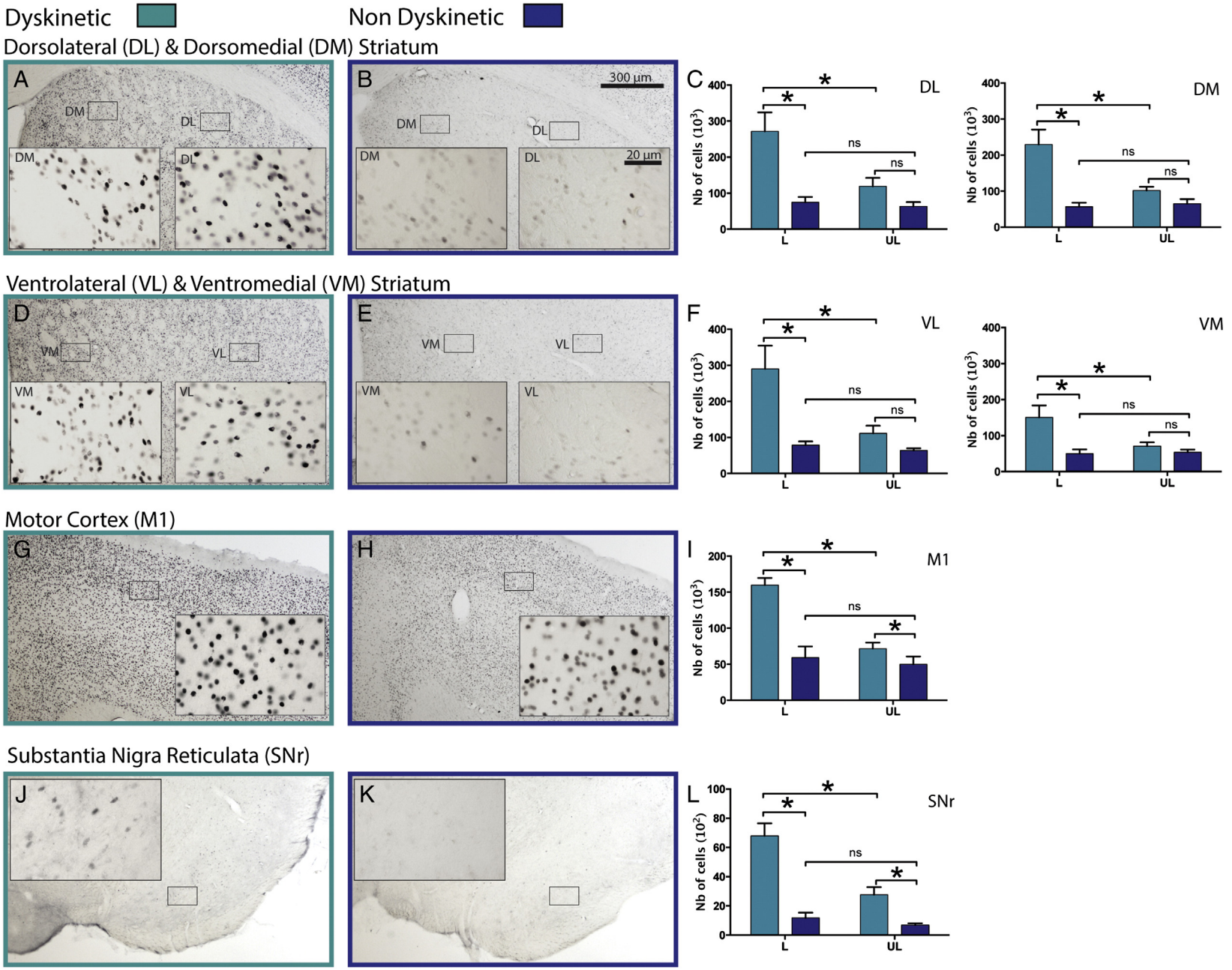
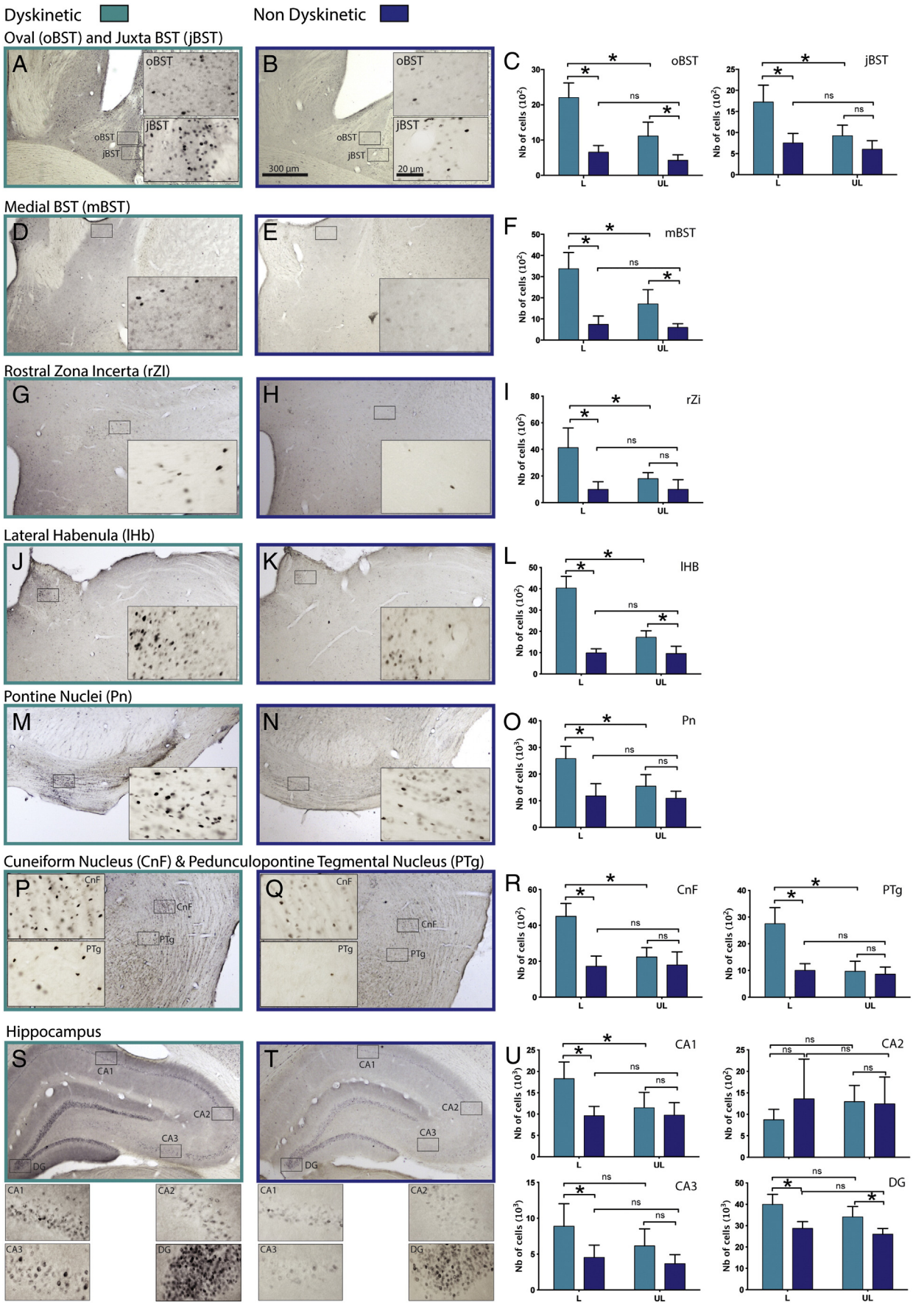


Fig. 4. Stereological counting of Zif268 immuno-positive cells in the cortico-basal ganglia motor loop in dyskinetic (light blue) and non-dyskinetic (dark blue) 6-OHDA-lesioned rats. Representative examples of staining, scale bar 300 µm (with an inset magnification, scale bar 20 µm), are shown on the left side while quantitative results are displayed on the right side (shown as mean ± SD; *$p < 0.05$). A–C dorsolateral (DL) $F_{[1.16]} = 27.13$, $p < 0.001$ and dorsomedian (DM) $F_{[1.16]} = 44.31$, $p < 0.001$ striatum; D–F ventrolateral (VL) $F_{[1.16]} = 28.87$, $p < 0.001$ and ventromedian (VM) $F_{[1.16]} = 24.66$, $p < 0.001$ striatum; G–I, M1 motor cortex $F_{[1.16]} = 59.01$, $p < 0.001$; J–L, substantia nigra pars reticulata (SNr) $F_{[1.16]} = 55.05$, $p < 0.001$.


Dyskinetic
Non Dyskinetic
Oval (oBST) and Juxta BST (jBST)
A
oBST
jBST
B
300 µm
20 µm
C
oBST
jBST
Nb of cells (10²)
ns
L
UL
Medial BST (mBST)
D
E
F
mBST
Rostral Zona Incerta (rZI)
G
H
I
rZi
Lateral Habenula (lHb)
J
K
L
lHB
Pontine Nuclei (Pn)
M
N
O
Pn
Nb of cells (10³)
Cuneiform Nucleus (CnF) & Pedunculopontine Tegmental Nucleus (PTg)
P
CnF
PTg
Q
R
CnF
PTg
Hippocampus
S
CA1
CA2
CA3
DG
T
U
CA1
CA2
CA3
DG

The rat model of LID, interesting and insightful as it is (Cenci et al., 2002), is a unilateral model of PD and LID. A number of changes can therefore affect the unlesioned hemisphere, either as a consequence of the contralateral lesion or subsequently to the L-DOPA treatment. Accordingly, we report numerous increased IEG expressions on the unlesioned side of dyskinetic rats compared to the unlesioned side of non-dyskinetic ones in structures both inside and outside the basal ganglia. Thus, despite an intact dopaminergic system, the unlesioned side of dyskinetic rats is transcriptionally more active than the lesioned and unlesioned side of non-dyskinetic rats and significantly less active than the lesioned side of dyskinetic rats regarding the 4 IEGs assessed in this study. These results highlight that the unlesioned side may not be considered as a non-affected reference when assessing gene expression in the dyskinetic 6-OHDA hemiparkinsonian rat model.

The number of IEG-immunopositive cells in various structures, both within and outside the basal ganglia, nicely correlates with the severity of LID (Fig. 9). Within the basal ganglia, the number of ΔFosB immuno-positive cells correlated with the intensity of LID in the dorsolateral ($r^2 = 0.87$, $P < 0.001$) and in the ventrolateral ($r^2 = 0.90$, $P < 0.001$) striatum (Fig. 9C), a finding consistent with previous reports (Andersson et al., 1999; Cenci and Konradi, 2010; Sgambato-Faure et al., 2005; Valastro et al., 2007) while the present is the first demonstration using stereological methods. Outside the basal ganglia, 2 nuclei of the BST showed significant correlations between the intensity of LID and, respectively, the number of ΔFosB-positive cells ($r^2 = 0.91$, $P < 0.001$) for the oBST and FRA2-positive cells for the jBST ($r^2 = 0.65$, $P < 0.05$) (Fig. 9D). The BST is a cluster of nuclei that receive robust monoaminergic inputs featuring serotonin (5-HT), noradrenealine (NA, or norepinephrine) and dopamine (DA) (Phelix et al., 1992). The BST DA inputs originate from the ventral tegmental area (VTA), the periaqueductal grey region and the retrorubral field. They form a fairly diffuse input to the dorsolateral BST with dense DA terminal fields in the oBST and the jBST (Freedman and Cassell, 1994; Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006). More importantly, in the oBST, recent data indicate that exogenous DA can reduce the inhibitory synaptic transmission in a D2 like dopamine receptor-dose-dependent manner in a brain slice of drug-naïve rats (Krawczyk et al., 2011) and that cocaine maintenance rats display an overexpression of D1R (Krawczyk et al., 2013), underlying the hypothesis of a potential role of the BST in LID.

In the epithalamus, the lHb showed a significant correlation between the LID intensity and the number of ARC immuno-positive cells ($r^2 = 0.85$, $P < 0.001$) (Fig. 9G). The medial part of the lHb is primarily innervated by the limbic system (Herkenham and Nauta, 1977; Hikosaka et al., 2008) while the lateral part receive basal ganglia afferents, especially from the internal part of the globus pallidus (GPi) (Hong and Hikosaka, 2008), the main output structure of the basal ganglia. The lHb projects mainly to the monoaminergic brain regions like the VTA, the SNc, the serotoninergic dorsal and medial raphe and also to the cholinergic laterodorsal tegmentum (Bernard and Veh, 2012; Geisler and Trimble, 2008; Hikosaka et al., 2008). Thus, the lHb acts as a junction connecting the limbic system and the basal ganglia to the monoaminergic centres. As the false transmitter hypothesis involving the serotoninergic system in LID pathophysiology addresses the presynaptic component of LID pathophysiology (Carta and Bezard, 2011; Navailles et al., 2010), the efferent connectivity of the lHb suggests that it may play a role in controlling serotoninergic output. Impaired lHb input would thus participate in the aberrant dopamine release from 5-HT terminals (Carta et al., 2007, 2008a,b; Navailles et al., 2011; Rylander et al., 2010).

In the brainstem, 2 nuclei, the Pn and CnF, displayed a significant correlation between LID intensity and, respectively, the number of Zif268 immuno-positive cells ($r^2 = 0.73$, $P < 0.01$) and FRA2 immuno-positive cells ($r^2 = 0.82$, $P < 0.001$) (Figs. 9JK). Both the Pn and the CnF receive afferents from the basal ganglia, i.e. from the STN (Wu and Hallett, 2013) and the SNr (Rolland et al., 2011), respectively. The Pn primarily projects to the cerebellum (Brodal, 1979, 1980) and, thus, could be considered as a relay structure from the basal ganglia through the STN (Bostan et al., 2010; Wu and Hallett, 2013). As the cerebellum is involved in fine tuning motor behaviour (Bastian, 2006; Thach et al., 1992; Timmann et al., 2010), it should not be surprising that a L-DOPA-induced modification of the Pn neurons' transcriptional activity could influence cerebellum-driven motor functions and impact LID pathophysiology. The CnF, known as a mesencephalic locomotor area, receives DA inputs (Rolland et al., 2009; Takakusaki et al., 2003). Although the origin of its dopaminergic innervation is still unclear, recent data indicate that MPTP monkeys undergo a dramatic decrease in CnF DA content (Rolland et al., 2009), providing information about a putative role in parkinsonian symptoms. Moreover, as the CnF projects back to the SNc (Watabe-Uchida et al., 2012), both CnF and SNc/SNr neurons form a direct loop that could play a role in motor-related behaviour, and hence support the involvement of the CnF in LID.

Conclusion

In conclusion, both motor and non-motor domains of cortico-subcortical loops showed significant correlations between the number of ΔFosB, ARC, FRA2 and Zif268 immunopositive cells and LID severity. A correlation does not necessarily imply a causal relationship but might reflect the concomitance of unrelated events. One should not therefore eliminate the possibility that these animals are experiencing other L-DOPA-induced side effects that were not investigated. Defining the precise role of these structures in LID pathophysiology now requires modulating the electrophysiological activity of these identified brain nuclei and assess the impact of such modulation upon motor behaviour in general and LID severity in particular.

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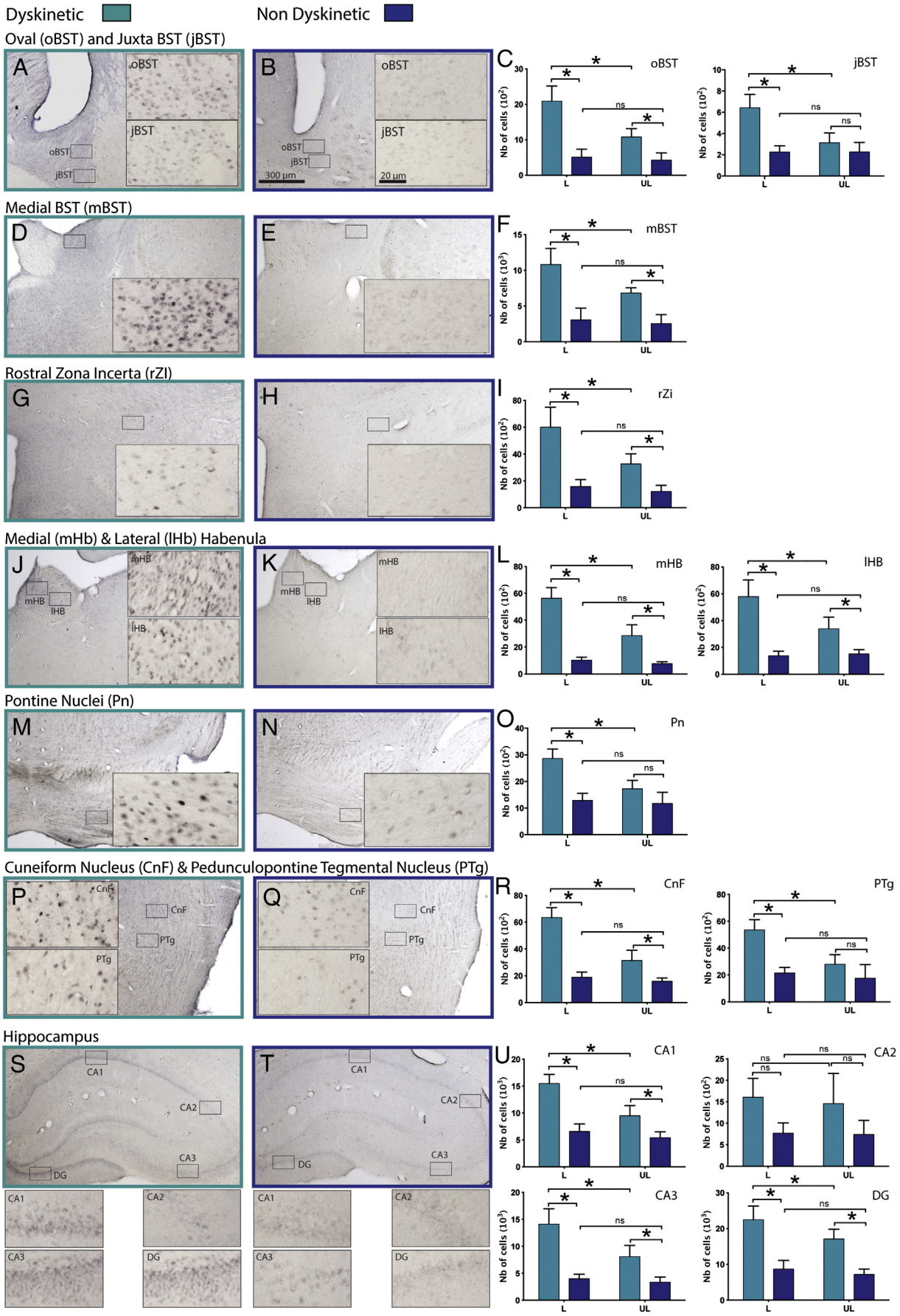
Fig. 5. Stereological counting of ΔFosB immuno-positive cells outside the basal ganglia in dyskinetic (light blue) and non-dyskinetic (dark blue) 6-OHDA-lesioned rats. Representative examples of staining, scale bar 300 μm (with an inset magnification, scale bar 20 μm), are shown on the left side while quantitative results are displayed on the right side (shown as mean ± SD; *$p < 0.05$). A–C, oval (oBST) $F_{[1.16]} = 9.626$, $p < 0.01$ and juxta (jBST) $F_{[1.16]} = 6.750$, $p < 0.05$ bed nucleus of the stria terminalis (BST); D–F, medial BST (mBST) $F_{[1.16]} = 9.438$, $p < 0.01$; G–I, rostral zona incerta (rZI) $F_{[1.16]} = 8.398$, $p < 0.05$; J–L, lateral habenula (lHb) $F_{[1.16]} = 47.5$ $p < 0.001$; M–O, pontine nuclei (Pn) $F_{[1.16]} = 6.657$, $p < 0.05$; P–R, cuneiform nucleus (CnF) $F_{[1.16]} = 16.90$, $p < 0.001$ and pedunculopontine tegmental nucleus (PTg) $F_{[1.16]} = 20.93$, $p < 0.001$; S–U, hippocampus CA1 $F_{[1.16]} = 5.807$, $p < 0.05$, CA2 $F_{[1.16]} = 0.9688$, $p = 0.33$, CA3 $F_{[1.16]} = 11.69$, $p < 0.05$ and dentate gyrus (DG) $F_{[1.16]} = 29.87$, $p < 0.001$.


Dyskinetic
Non Dyskinetic
Oval (oBST) and Juxta BST (jBST)
A
oBST
jBST
B
oBST
jBST
300 µm
20 µm
C
oBST
jBST
Nb of cells (10²)
L
UL
ns
Medial BST (mBST)
D
E
F
mBST
Nb of cells (10³)
Rostral Zona Incerta (rZI)
G
H
I
rZi
Nb of cells (10²)
Medial (mHb) & Lateral (lHb) Habenula
J
mHB
lHB
K
L
mHB
lHB
Nb of cells (10²)
Pontine Nuclei (Pn)
M
N
O
Pn
Nb of cells (10²)
Cuneiform Nucleus (CnF) & Pedunculopontine Tegmental Nucleus (PTg)
P
CnF
PTg
Q
R
CnF
PTg
Nb of cells (10²)
Hippocampus
S
CA1
CA2
CA3
DG
T
U
CA1
CA2
CA3
DG
Nb of cells (10³)
Nb of cells (10²)

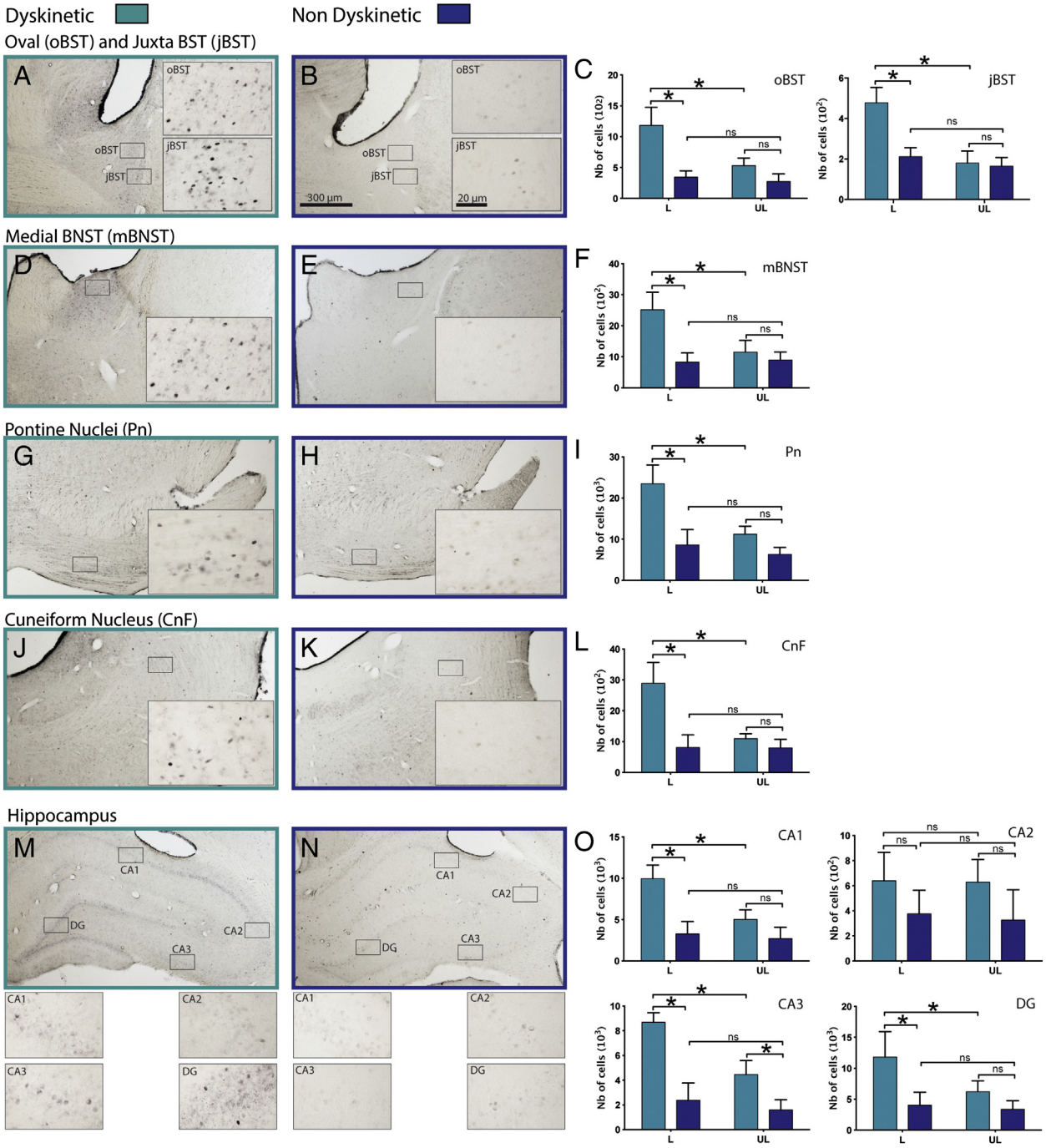


Fig. 7. Stereological counting of FRA2 immuno-positive cells outside the basal ganglia in dyskinetic (light blue) and non-dyskinetic (dark blue) 6-OHDA-lesioned rats. Representative examples of staining, scale bar 300 µm (with an inset magnification, scale bar 20 µm), are shown on the left side while quantitative results are displayed on the right side (shown as mean ± SD; *$p < 0.05$). A–C, oval (oBST) $F_{[1.16]} = 13.42$, $p < 0.01$ and juxta (jBST) $F_{[1.16]} = 24.23$, $p < 0.001$ bed nucleus of the stria terminalis (BST); D–F, medial BST (mBST) $F_{[1.16]} = 16.94$, $p < 0.01$; G–I, pontine nuclei (Pn) $F_{[1.16]} = 12.18$, $p < 0.01$; J–L, cuneiform nucleus (CnF) $F_{[1.16]} = 22.13$, $p < 0.001$; M–O, hippocampus CA1 $F_{[1.16]} = 11.83$, $p < 0.01$, CA2 $F_{[1.16]} = 0.042$, $p < 0.84$, CA3 $F_{[1.16]} = 13.18$, $p < 0.01$ and dentate gyrus (DG) $F_{[1.16]} = 4.661$, $p < 0.05$.

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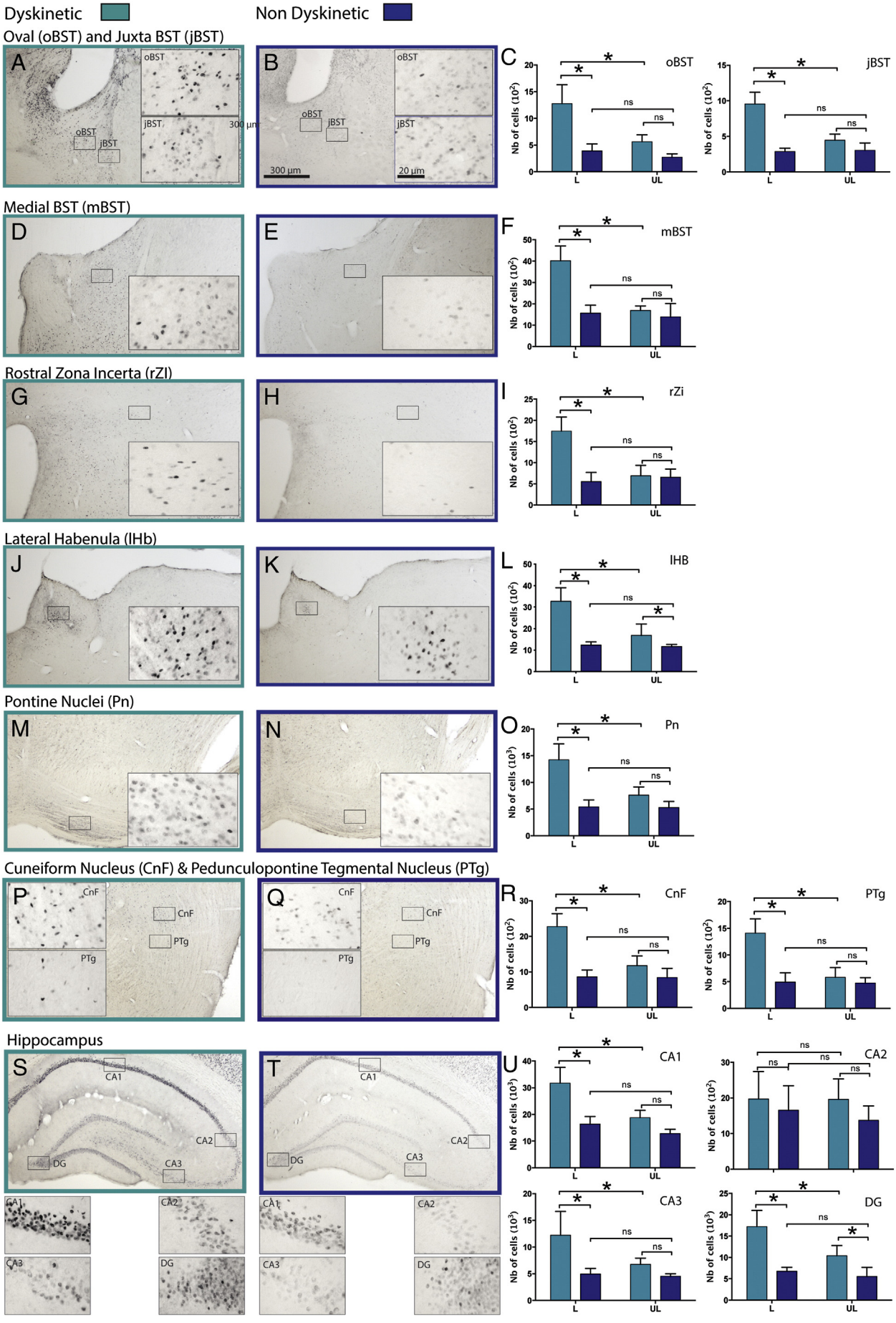
Fig. 6. Stereological counting of ARC immuno-positive cells outside the basal ganglia in dyskinetic (light blue) and non-dyskinetic (dark blue) 6-OHDA-lesioned rats. Representative examples of staining, scale bar 300 µm (with an inset magnification, scale bar 20 µm), are shown on the left side while quantitative results are displayed on the right side (shown as mean ± SD; *$p < 0.05$). A–C, oval (oBST) $F_{[1.16]} = 13.04$, $p < 0.01$ and juxta (jBST) $F_{[1.16]} = 15.19$, $p < 0.001$ bed nucleus of the stria terminalis (BST); D–F, medial BST (mBST) $F_{[1.16]} = 6.190$, $p < 0.05$; G–I, rostral zona incerta (rZI) $F_{[1.16]} = 8.729$, $p < 0.01$; J–L, medial (mHb) $F_{[1.16]} = 23.53$, $p < 0.001$ and lateral habenula (lHb) $F_{[1.16]} = 13.08$, $p < 0.01$; M–O, pontine nuclei (Pn) $F_{[1.16]} = 11.16$, $p < 0.01$; P–R, cuneiform nucleus (CnF) $F_{[1.16]} = 31.27$, $p < 0.001$ and pedunculopontine tegmental nucleus (PTg) $F_{[1.16]} = 10.50$, $p < 0.01$; S–U, hippocampus CA1 $F_{[1.16]} = 12.24$, $p < 0.01$, CA2 $F_{[1.16]} = 0.082$, $p = 0.78$, CA3 $F_{[1.16]} = 10.42$, $p < 0.01$ and dentate gyrus (DG) $F_{[1.16]} = 8.112$, $p < 0.05$.


Dyskinetic
Non Dyskinetic
Oval (oBST) and Juxta BST (jBST)
A
oBST
jBST
300 µm
B
oBST
jBST
300 µm
20 µm
C
oBST
jBST
Nb of cells (10²)
ns
L
UL
Medial BST (mBST)
D
E
F
mBST
Rostral Zona Incerta (rZI)
G
H
I
rZi
Lateral Habenula (lHb)
J
K
L
lHB
Pontine Nuclei (Pn)
M
N
O
Pn
Nb of cells (10³)
Cuneiform Nucleus (CnF) & Pedunculopontine Tegmental Nucleus (PTg)
P
CnF
PTg
Q
R
CnF
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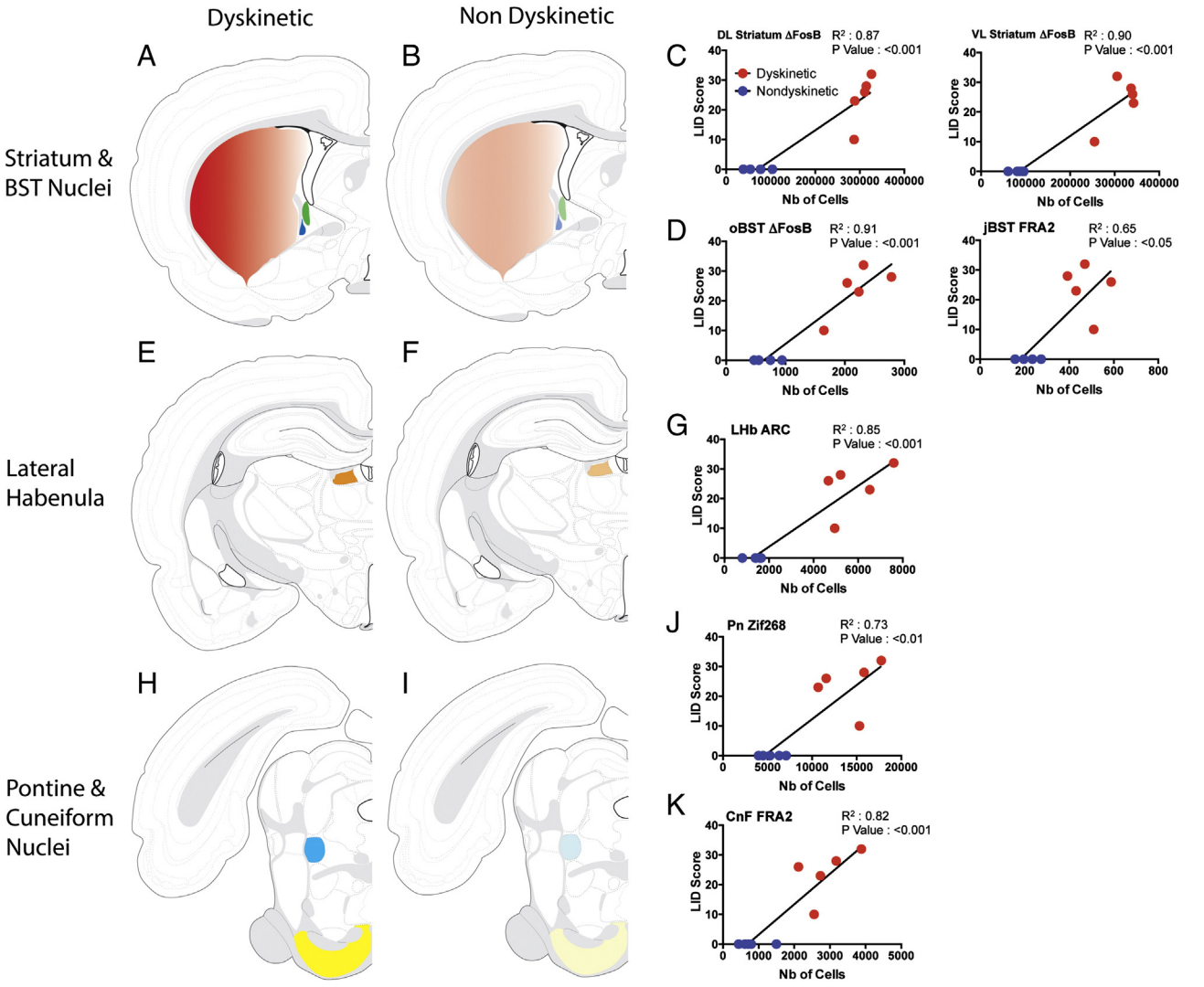

Fig. 9. Correlation between the number of ΔFosB, ARC, FRA2 and Zif268 immuno-positive cells in certain brain nuclei and LID severity (sum of the axial, limb, and orolingual AIMs (maximal score for each observation, 12; total score per session, 48)). Atlas-based localization of brain area allows displaying relative difference in number of IEG-immunopositive cells on the lesioned side between dyskinetic and non-dyskinetic rats. The darker the colour the greater the number of immunopositive cells (striatum: red; oval and juxta bed nucleus of the stria terminalis: green and dark blue, respectively; lateral habenula: orange; pontine and cuneiform nuclei: yellow and light blue, respectively). Specific IEG correlations between number of IEG immunopositive cells and LID severity are displayed on the right side. A–D, dorsolateral (DL), ventrolateral (VL) striatum, oval (oBST) and juxta (jBST) bed nucleus of the stria terminalis (BST); E–G, lateral habenula (lHb); H–K, pontine (Pn) and cuneiform (CnF) nuclei.

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Fig. 8. Stereological counting of Zif268 immuno-positive cells outside the basal ganglia in dyskinetic (light blue) and non-dyskinetic (dark blue) 6-OHDA-lesioned rats. Representative examples of staining, scale bar 300 μm (with an inset magnification, scale bar 20 μm), are shown on the left side while quantitative results are displayed on the right side (shown as mean ± SD; *$p < 0.05$). A–C, oval (oBST) $F_{[1.16]} = 11.05$, $p < 0.01$ and juxta (jBST) $F_{[1.16]} = 29.82$, $p < 0.001$ bed nucleus of the stria terminalis (BST); D–F, medial BST (mBST) $F_{[1.16]} = 22.32$, $p < 0.001$; G–I, rostral zona incerta (rZI) $F_{[1.16]} = 26.74$, $p < 0.001$; J–L, lateral habenula (lHb) $F_{[1.16]} = 16.36$, $p < 0.001$; M–O, pontine nuclei (Pn) $F_{[1.16]} = 14.87$, $p < 0.01$; P–R, cuneiform nucleus (CnF) $F_{[1.16]} = 19.07$, $p < 0.001$ and pedunculopontine tegmental nucleus (PTg) $F_{[1.16]} = 22.99$, $p < 0.001$; S–U, hippocampus CA1 $F_{[1.16]} = 8.402$, $p < 0.05$, CA2 $F_{[1.16]} = 0.2381$, $p = 0.63$, CA3 $F_{[1.16]} = 5.661$, $p < 0.05$ and dentate gyrus (DG) $F_{[1.16]} = 6.205$, $p < 0.05$.

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2. Publication 2: Selective inactivation of striatal FosB/ΔFosB-expressing neurons alleviates L-Dopa induced dyskinesia

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In the previous study, we identified several brain nuclei outside of the basal ganglia displaying a significant correlation between IEG expression and LID severity. Unravelling the precise role of these structures in LID pathophysiology now requires a selective modulation of their electrophysiological activity and the assessment of the impact of such a modulation upon LID severity. To do so, we used the Daun02 inactivation method allowing us to selectively decrease the neuronal activity of IEG-expressing neurons in selected brain nuclei. However, this inactivation method is new in the context of LID. In this study, we validate this innovative method in a structure already known to be involved in LID. Lessons from the past were two-fold: (i) the striatum is undoubtedly central in LID pathophysiology and (ii) it displays an increased expression of ΔFosB both in dyskinetic rodents and macaque. In addition, RNA interference against ΔFosB mRNA decrease LID severity, underlying the functional impact of ΔFosB on LID. In the present study, we selectively inhibited the electrical activity of striatal FosB/ΔFosB-expressing neurons with the Daun02 method. Interestingly, Daun02 injection induces a decrease in LID severity without affecting the benefits of L-Dopa therapy both in dyskinetic 6-OHDA-lesioned rats and MPTP-treated macaques. Taken together, our results (i) validate the Daun02 inactivation method in the LID field and (ii) demonstrate, for the first time, the casual link between the electrical activity of striatal FosB/ΔFosB-expressing neurons and LID severity.

Selective inactivation of striatal FosB/ΔFosB-expressing neurons alleviates L-Dopa-induced dyskinesia

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Keywords: dyskinesia / Parkinson's disease / Daun02 / electrophysiology / monkey / FosB

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Abstract word count: 221

Number of figures: 4

Number of tables: 0

Running title: Dyskinesia and deltaFosB

Abstract

Background: ΔFosB is a surrogate marker of L-Dopa-induced dyskinesia (LID), the unavoidable disabling consequence of Parkinson's disease (PD) L-dopa long-term treatment. However, the relationship between the electrical activity of FosB/ΔFosB-expressing neurons and LID manifestation is unknown.

Methods: We used the Daun02 prodrug-inactivation method associated with lentiviral expression of ß-galactosidase under the control of the FosB promoter to investigate a causal link between the activity of FosB/ΔFosB-expressing neurons and dyskinesia severity in both rat and monkey models of PD and LID. Whole-cell recordings of medium spiny neurons (MSNs) were performed to assess the effects of Daun02 and daunorubicin on neuronal excitability

Results: We first show that daunorubicin, the active product of Daun02 metabolism by ß-galactosidase, decreases the activity of MSNs in rat brain slices, and that Daun02 strongly decreased the excitability of rat MSNs primary cultures expressing ß-galactosidase upon D1 dopamine receptor stimulation. We then demonstrate that the selective, and reversible, inhibition of FosB/ΔFosB-expressing striatal neurons with Daun02 decreases the severity of LID while improving the beneficial effect of L-Dopa.

Conclusions: These results establish that FosB/ΔFosB accumulation ultimately results in altered neuronal electrical properties sustaining maladaptive circuits leading not only to LID, but also to a blunted response to L-Dopa. These findings further reveal that targeting dyskinesia can be achieved without reducing the antiparkinsonian properties of L-Dopa when specifically inhibiting FosB/ΔFosB-accumulating neurons.

Introduction

L-Dopa-induced dyskinesia (LID) is a debilitating side effect of chronic dopamine replacement therapy in Parkinson's disease (PD). Among the molecular alterations underlying LID (1, 2), accumulation of FosB and of its truncated splice variant ΔFosB have been identified as a surrogate markers of LID in experimental models of PD (3). Molecular interference with FosB or ΔFosB using either antisense oligonucleotides or a dominant negative inhibitor ΔJunD reduces LID (4, 5), demonstrating that disruption of transcriptional regulation linked to FosB and ΔFosB underlies the development of LID. However, the precise relationships between FosB/ΔFosB accumulation and neuronal activity sustaining LID remain unknown since no causal link between the electrical activity of FosB/ΔFosB-expressing neurons and LID has ever been established. To test the hypothesis that the intrinsic activity of these neurons directly mediates this side-effect of dopamine replacement therapy in PD, we used FosB as a molecular marker of LID to selectively express ß-galactosidase in FosB/ΔFosB-expressing neurons and assessed the role of these neurons in rat and monkey models of LID in PD (6-8) by inhibiting their electrical activity using Daun02-inactivation (9-12).

Material and Methods

Study approval

Experiments were performed in accordance with the European Union directive of September 22, 2010 (2010/63/EU) on the protection of animals used for scientific purposes. Experiments were approved by the Institutional Animal Care and Use Committee of Bordeaux (CE50) under the license numbers 5012099-A (rats) and 50120102-A (Monkeys). Monkey experiments were performed in an AAALAC-accredited facility following acceptance of study design by the Institute of Lab Animal Science (Chinese Academy of Science, Beijing, China) IACUC.

FosB–LacZ lentivirus

A 1253 bp fragment upstream of the transcription initiation site of the rat FosB genomic DNA was cloned by PCR with the following primers: sense, 5'-caccggatcccacagaccctccaacttctc-3', antisense, 5'-ccggctagcttccctgggcacaggggggcccctgtgaccacgctgaggtctt-3'. PCR products were purified, digested with BamHI/NheI, and subcloned into a LacZ reporter plasmid (13). The

plasmid was subcloned into the lentiviral vector (LV) pRRLSIN-cPPT-PGK-MCS-WPRE. All constructs were verified by sequencing.

LV production was performed at INSERM E217/Vectorology Platform-IFR 66 by transfection with a three viral vector system, mock (with the different inserts), pCMV-Δ8-9 (encapsidation plasmid), VSV-G (cDNA encoding the envelope glycoprotein of vesicular stomatitis virus) in FT-HEK293 cells. Lentiviral supernatants were concentrated by centrifugation concentration filter (centricon) with a final titer of $1.18.10^9$ infectious particles/ml.

Validation on rat striatal primary culture

Rat striatal cultures were prepared from E15 rat brains as previously described (14). Cells were plated on coverslips coated with 10 μg/mL poly-D-lysine and laminin at a density of 150000/dish. Striatal cultures were grown in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM glutamine, and 12.5 μM glutamate. Before seeding, coverslips were incubated with foetal calf serum-supplemented 20% Dulbecco's modified Eagle medium/F12 (1h at 37°C). After seeding, cells were kept at 37°C in a 5% CO2 incubator for 10 days. At 4 days *in vitro* (DIV4), part of the medium together with non-adherent cells and cell debris were removed and a culture medium with cytosine arabinofuranoside (0.5 μM) and without glutamate was added. Striatal neurons were transduced at DIV14 with 1μL of FosB-LacZ lentivirus. To induce neuronal FosB activation, neurons were incubated with 10 μM of the full D1R agonist SKF-82958 for 1h.

Cytochemical detection of β-galactosidase (β-gal)

Cultured cells were washed twice in PBS (pH=7.4), fixed with 4.0% paraformaldehyde for 5 minutes at room temperature, and incubated overnight at 37°C in freshly prepared staining buffer [1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 5 mM K3Fe[CN]6, 5mM K4Fe[CN]6, and 2 mM MgCl2 in PBS, pH 6.0]. Cells were washed with PBS, followed with methanol and examined at ×20 magnification.

Striatal cell excitability reduction by daunorubicin ex vivo

Nine male Sprague Dawley rats (3-4 weeks old) were used for experiments. Corticostriatal slices (200 μm) were prepared as described (15). Whole-cell current clamp recordings were performed from individual neurons, visualized with IR-DIC system (15) using a Multiclamp 700B amplifier (Axon Instruments). Pipettes (3-5 MΩ) were filled with: (in mM) K^+-

gluconate (125), NaCl (10), $CaCl_2$ (1.0), $MgCl_2$ (2.0), 1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA; 0.5), *N*-(2-hydroxyethyl)-piperazine-*N*-*s*-ethanesulfonic acid (HEPES; 19), guanosine triphosphate (GTP; 0.3), Mg-adenosine triphosphate (Mg-ATP; 1.0), pH=7.3. Data were acquired with pClamp 9.2 software (Molecular Device, USA) and analyzed offline (Clampfit 9.2, Molecular Devices, USA).

Daun02-inactivation of medium spiny neurons *in vitro*

Rat striatal cultures were prepared as described above. Recordings were performed 2 hrs after 9 mM daun02 incubation at room temperature (22-25°C) using pipettes (5-7 MΩ, World Precision Instruments, USA) filled with (in mM): K^+-gluconate (100), EGTA (1.1), HEPES (10), creatine phosphate (3), GTP (0.3), $CaCl_2$ (0.1) and $MgCl_2$ (5), pH=7.2. Data were recorded using a Multiclamp 700B amplifier by a computer running pClamp 10.2 software via a Digidata 1440A interface (Molecular Devices). Upon achieving whole cell, the resting membrane potential (RMP) was determined in current clamp mode. The amplifier mode was switched to voltage clamp with a baseline holding potential (Vh) of -70 mV. Pipettes series resistance (Rs) and capacitance (Cm) were tested. The amplifier was then switched to current clamp mode, membrane potential adjusted to -70 mV, and series of depolarizing current pulses of 2s duration were injected with 200 ms between each pusle. Action potentials and firing pattern of the neurons were monitored in response to depolarizing currents.

Behavioral experiments

Animals

Twelve male Sprague-Dawley rats (Charles River Laboratories, France) with water and regular rodent chow available *ad libitum* were used. All experiments were approved by the Ethical Committee of Bordeaux University. Two male macaques (Macaca fascicularis, Beijing, PR of China; aged 5±1 years; weight = 5.3±0.8 kg) housed in individual primate cages allowing visual contacts and interactions with monkeys housed in adjacent cages, under controlled conditions. Food and water were available *ad libitum* and animal care was supervised daily by veterinarians skilled in the healthcare and maintenance of nonhuman primates.

Rats experiments

Experimental parkinsonism was achieved with an unilateral injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB) as previously described (6, 16-18).

Surgeries were conducted under isoflurane anesthesia. Coordinates are given in mm relative to bregma and dura (19). Briefly, 30min after injection of desipramine + citalopram (Sigma-Aldrich; 20mg/kg and 1mg/kg respectively; ip) 2.5 μL 6-OHDA (Sigma-Aldrich; 3 μg/μL in 0.1% ascorbic acid) was injected in the right MFB (AP -3.6; ML +1.6; DV -7.5). Animals were injected with 10 μL FosB/LacZ LV in the right striatum (AP 0.5; ML +3.6; DV -4.7). Guide cannulas (Plastic One; 26 gauges) were implanted in 7 rats (AP 0.5; ML +3.6; DV -3.2) and cemented to the skull for subsequent Daun02 infusions. Rats were placed in recovery during 3 weeks with daily monitoring then underwent stepping test (20) to evaluate motor deficits. Only animals exhibiting >50% stepping deficits were retained for AIMs induction.

Animals then received 10 daily injections of L-Dopa/benserazide (Sequoia Research Products, 6mg/kg and 15mg/kg respectively; ip). On the last day, baseline AIMs were scored as previously described (6, 8, 16-18) using a validated rating scale assessing 3 items (axial, forelimb, orolingual dyskinesia) rated from 0 (absent) to 4 (continuous, severe, uninterrupted by sensory distraction) (21) to obtain a composite score (max 12pts). Ratings lasted 1min/rat at 30, 60, 90 and 120min post L-dopa. The 4 composite scores were added to obtain a cumulated score (max 48pts). L-Dopa-induced rotations were rated following the same schedule to obtain a cumulated score (max 16 pts).

On the 11th day, animals received a 6mg/kg L-dopa injection 1h before a 2 μL Daun02 injection (4 μg/μL in 5% DMSO, 5% Tween-80 in PBS at 0.5 μl/min) under light isoflurane anesthesia before being placed in their home cage for 3 days as described (10, 11). From the 4th day, all rats received a daily 6 mg/kg L-Dopa injection and AIMs were scored. Because 6 mg/kg L-Dopa induces marked dyskinesia in rats (16, 22) possibly reaching plateau levels, a 4 mg/kg dose was further tested. AIMs and rotation scores were then stabilized with 4 mg/kg L-Dopa during 5 consecutive days with a baseline rating on the last day. Using the same infusion procedure and after a 3-days rest period, animals received once-daily 4 mg/kg L-Dopa injection. AIMs and rotations were scored during 3 days. To ensure reversibility of Daun02-induced inactivation, 6 mg/kg L-Dopa was administered after 8 days wash-out and AIMs were evaluated. 1.5 hour after the last L-dopa administration, rats were euthanized with a lethal injection of chloral hydrate (600mg/kg, VWR) and perfused with 2% paraformaldehyde + 0.3% glutaraldehyde for histological analysis.

Monkey experiments

Animals were first rendered parkinsonian with MPTP-hydrochloride (0.2mg/kg, i.v., Sigma) dissolved in saline as previously described (23). Daily (9 a.m.) assessment of parkinsonism

was performed in home cages for 30 min by two blinded observers using a validated rating scale (23) assessing tremor, general level of activity, body posture (flexion of spine), vocalization, freezing and frequency of arm movements and rigidity (for each upper limb). Following stabilization of the MPTP-induced syndrome (3 months), animals received twice-daily 20mg/kg L-Dopa p.o. for three months and developed severe and reproducible dyskinesia, presenting choreic–athetoid (characterized by constant writhing and jerking motions), dystonic and sometimes ballistic movements (large-amplitude flinging, flailing movements). Once animals were stably dyskinetic, striatal stereotactic delivery of viral vector was conducted under isoflurane anesthesia as previously described (6, 8). Horsley-Clarke stereotaxic technique coupled with ventriculography were used to determine the position of left and right putamen. A total volume of 100 μL of FosB/LacZ lentivirus was injected bilaterally into each animal (50 μL per side at 2 rostrocaudal and 2 dorsoventral sites (AP -1 and 1; ML +/- 14; DV 0 and 3 from anterior commissura (AC)) with a Hamilton syringe mounted into a microinjector system (Kopf, California) (6, 8). Guide cannulas (AP 0; ML +/- 14, DV 7 from AC) were cemented to the skull as previously described (24-26).

Monkeys' behavior was recorded OFF and ON L-dopa before, while being exposed (3-5 days after intrastriatal injection) and after (7 days after intrastriatal injection) of Daun02 (25 μl per hemisphere at 2 μl/min, 4 μg/μL dissolved in 5% DMSO, 5% Tween-80 in PBS under light isoflurane anesthesia). Each time, they were first recorded in the OFF state for 60 min in an observation cage (dimensions - 1.1m x 1.5m x 1.1m). L-dopa was then administered, and the monkeys' behavior was recorded for a further 240 min in the observation cage. The total duration of observation was 300 min including drug administration. The parkinsonian condition (and its reversal) was assessed on a parkinsonian monkey rating scale using videotape recordings of monkeys. A score of 0 corresponds to a normal animal and a score above 6 to a parkinsonian animal. The severity of dyskinesia was rated using the Dyskinesia Disability Scale (27) as previously described (6, 8, 28-31): 0, dyskinesia absent; 1, mild, fleeting, and rare dyskinetic postures and movements; 2, moderate, more prominent abnormal movements, but not interfering significantly with normal behavior; 3, marked, frequent and, at times, continuous dyskinesia intruding on the normal repertoire of activity; or, 4, severe, virtually continuous dyskinetic activity replacing normal behavior and disabling to the animal. The duration of anti-parkinsonian action, i.e. on-time, was defined as the number of minutes for which bradykinesia was absent i.e. score equal to zero. In addition, the duration of on-time associated with dyskinesia of varying severity was defined as follows; "good" quality on-time

represents the number of minutes for which bradykinesia was zero whilst dyskinesia was either absent or of mild or moderate severity (0-2).

Transduction volumes

Striatal stereotaxic infusions (n=6) of 10μL lentiviral vectors driving the expression of β-galactosidase under a constitutive neuronal promoter were conducted in rats as described above. After tissue processing for X-gal staining, coronal sections were sampled throughout the striatum and the transduction volume was calculated with the Cavalieri's principle using Mercator image analysis system (Explora Nova, France). The three-dimensional reconstruction of the transduction volume was achieved using Map3D software (Explora Nova, France).

Histological analysis

50 μm-thick free-floating coronal sections from rat and macaques were collected and processed for tyrosine hydroxylase (MAB318, Milipore), ΔFosB/D1R (sc-48, Santa-Cruz and D2944, Sigma, respectively), and ΔFosB/β-galactosidase (ab11959, abcam and AB1211-5MG, Millipore immunohistochemistry as previously described (32). Free-floating sections were incubated for X-gal staining for 4h with [1mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 5mM K3Fe[CN]6, 5mM K4Fe[CN]6, and 5mM MgCl2 in PBS] and counterstained with neutral red.

Data analysis

Electrophysiological data were analyzed using 2-way analysis of variance with repeated measures (RM ANOVA) and paired t-tests. For behavioral data, (2-way RM ANOVA) were run with Bonferroni *post-hoc* tests. All data are presented as mean ± SEM with a threshold for statistical significance at $p<0.05$.

Results

The Daun02 inactivation method has been originally designed for the treatment of human malignancies (33). It consists into the local administration of the prodrug Daun02 converted into daunorubicin by β-galactosidase, readily expressed in mammalian cells previously transduced with the E. coli LacZ gene under the control of a cell-specific promoter (10-12). Daunorubicin has been shown to reduce calcium ion (Ca^{2+})-dependent action potentials in neuroblastoma cells (9). Despite previous usage in the prefrontal cortex (11, 12) or in the

nucleus accumbens (10) the electrophysiological demonstration of striatal medium spiny neurons (MSNs) inactivation using daunorubicin has yet to be demonstrated. We first showed that daunorubicin significantly reduces MSNs activity elicited by depolarizing current steps in rat brain slices ($p<0.01$, **Fig 1A, B**). We further validated that Daun02 strongly decreased the excitability of rat MSNs primary cultures constitutively expressing LacZ ($p<0.01$ **Fig 1C, D**), establishing the ability of LacZ expressing-cells to convert Daun02 into daunorubicin to mediate inactivation. Recordings performed after Daun02 washout confirmed the reversibility of Daun02-induced inactivation (**Fig 1D**). Differences in cellular maturation and channel composition may account for the different resting membrane potential, input resistance and therefore firing patterns of cultured MSNs, as compared to those recorded from slices (34, 35). In addition, primary MSN cultures are partially deafferented, a feature that may also account for different firing patterns as a result of the development of neuronal circuits (36).

We then developed a FosB-lacZ lentivirus and demonstrated *in-vitro* that transduced rat MSNs stimulated with the D1R agonist SKF-82958, that activates transcription of ΔFosB (37, 38), expressed β-galactosidase (**Fig 2**). Thus, we could selectively inactivate β-galactosidase transduced neurons co-expressing FosB/ΔFosB upon dopaminergic stimulation.

The FosB-lacZ lentivirus was injected *in-vivo* to evaluate the role of FosB-expressing neurons on abnormal involuntary movements (AIMs) in 6-hydroxydopamine-lesioned rats (6, 8, 18, 39), the rodent analog of LID. After the establishment of stable AIMs with a therapeutic dose of 6 mg/kg L-Dopa (6, 8, 16-18), a single intrastriatal administration of Daun02 significantly decreased AIMs compared with control rats during 2 days (RM ANOVA; Group x Day: $F_{[6, 60]}=2.64$; $p<0.05$; Bonferroni: $p<0.05$ for all; **Fig 3A**). In Daun02-treated animals, AIMs reduction lasted 3 days compared with their baseline scores (-25 %, -29 % and -24 % respectively; Bonferroni: $p<0.05$ for all; **Fig 3A**), in keeping with previous demonstration of Daun02-mediated behavioral span (10). In addition, Daun02 increased rotational behavior on the first day compared with control rats (+66 %; RM ANOVA; Group x Day: $F_{[6, 60]}=3.55$; $p<0.01$; Bonferroni: $p<0.05$; **Fig 3B**). When the L-Dopa dose was reduced to 4 mg/kg, Daun02 strongly decreased AIMs compared with control rats during 2 days (RM ANOVA; Group x Day: $F_{[3, 27]}=4.63$; $p<0.05$; Bonferroni: $p<0.01$ for all). AIMs reduction lasted 3 days compared with baseline levels (-53 %, -63 % and -45 % respectively; Bonferroni: $p<0.05$ for all; **Fig 3A**). At this dose, no change in rotational behavior was observed (**Fig 3B**). Animals were then rebaselined with 6 mg/kg L-Dopa and retrieved their original AIMs scores, demonstrating the reversibility of Daun02-inactivation. Double immunofluorescence for FosB/ΔFosB and D1R further highlighted that FosB/ΔFosB accumulation occurred both in

D1-positive and D1-negative neurons in the dorsolateral striatum (**Fig 3C**) (40). X-gal staining revealed that 18.3 % of the striatum was transduced by the construct (**Fig 4A, B**). Double immunofluorescence for β-galactosidase and FosB/ΔFosB confirmed the selective induction of β-galactosidase expression in FosB/ΔFosB-positive neurons (**Fig 4C**).

The data above indicate that Daun02-mediated reduction of FosB/ΔFosB-expressing neuron excitability significantly attenuates AIMs in 6-OHDA rats. In an effort to translate these findings into a clinically relevant context, we set out to determine (i) whether such approach can revert already established dyskinesias in an animal model that better recapitulates the human condition and (ii) whether the therapeutic effect can also be seen. We thus investigated the behavioral impact of the Daun02 inactivation method in the gold standard experimental model of LID, the MPTP-lesioned L-dopa–treated macaque monkey (6, 8, 30, 31, 41). Two L-dopa–treated dyskinetic macaques received the FosB-lacZ lentivirus in the motor putamen (6, 8, 42). Parkinsonian disability scores in both the OFF (before L-dopa administration) and ON states (after L-dopa administration), and LID scores in the ON state were indistinguishable between observations made before and 8 weeks after the intrastriatal delivery of FosB-lacZ lentivirus. When injected in the putamen of these monkeys, Daun02 significantly decreased the dyskinesia score (paired t-test; $p<0.05$; **Fig 3D**) without affecting the disability score (**Fig 3E**), resulting in a significantly increased 'good on-time' period (paired t-test; $p<0.05$; **Fig 3F**). Animals returned to their presurgery dyskinesia (**Fig 3D)** and disability (**Fig 3E)** scores 4 days later. Even though LID were not fully abolished, the magnitude of Daun02 effect for each animal (≥50% reduction, Fig 3D) together with a delay in the onset of dyskinesia (Figure 3D) represent clinically relevant effects. Taken together, these results indicate that Daun02-mediated inactivation of FosB/ΔFosB MSNs diminishes LID severity (**Fig 3D**), not only without reducing the positive effects of L-dopa on parkinsonian motor scores (**Fig 3E)** but also by allowing a longer good on-time (**Fig 3F),** the primary endpoint in most antidyskinesia clinical trials.

Discussion

Seminal studies evaluating metabolic changes in the basal ganglia have suggested that hyperactivity of the direct pathway sustains dyskinesia (43, 44). Electrophysiological studies further proposed a modified firing pattern involved in LID (45, 46). Marked abnormalities in neuronal activity and long-lasting molecular mechanisms prime and/or sustain LID (47). Among them, striatal FosB/ΔFosB accumulates in PD patients (48) and correlates with LID

severity both in rat and monkey models of PD (4, 5). Molecular interference studies further highlighted a causal link between ΔFosB and LID apparition (5) or expression (4). Despite marked progress in the understanding of the molecular mechanisms underlying LID, the links between this debilitating side-effect and the activity of neuronal populations displaying such molecular alterations remain poorly understood. Using FosB as a molecular marker of LID, we therefore aimed to investigate such relationships by selectively silencing the electrical activity of FosB/ΔFosB-expressing neurons in the motor striatum.

In PD patients, the presence of LID is inevitably associated with a decreased duration and/or magnitude of the therapeutic benefit of L-Dopa (49, 50). In addition, in pre-clinical models, most anti-dyskinetic drugs can negatively affect the duration and/or magnitude of the therapeutic effect of L-Dopa, highlighting their lack of strict selectivity towards the underlying mechanisms of LIDs (51, 52). Here, the selective silencing of FosB/ΔFosB-expressing neurons induced a reduction of LID together with an increased rotational behavior in rats and with an increase in good on-time period without changes in disability scores in primates. This dual effect establishes a dichotomous role for FosB/ΔFosB expressing neurons since our results demonstrate that their activity not only mediates LID but also inherently blunts the antiparkinsonian effect of L-Dopa.

LIDs derive from sensitized D1 receptors due to chronic L-Dopa stimulation (6, 53). Recent studies showed that ΔFosB overexpression in accumbal D1-expressing MSNs modulates synaptic properties by increasing spine density and modifying synaptic strength leading to increased cocaine-induced locomotion (54, 55). Conversely, reducing ΔFosB signaling prevents these morphological and behavioral modifications, suggesting that ΔFosB accumulation ultimately results in altered neuronal activity, leading to sensitized behaviors (56). Here, by selectively inactivating these FosB/ΔFosB -expressing neurons in maladaptive basal ganglia loops, we transiently reinstated the so-called 'honey-moon' period of L-Dopa treatment. Altogether, these results demonstrate that the activity of neurons underlying this side-effect of dopamine replacement therapy is also responsible for the loss of therapeutic benefit and therefore identify reduction of activity of FosB/ΔFosB-expressing neurons by any means as a highly specific procedure for counteracting LIDs without decreasing the therapeutic effect of dopamine replacement therapy. Strategies aiming to prevent the constitution of maladaptive circuits by targeting FosB/ΔFosB-expressing neurons represent promising avenues to preclude the development of LID while maintaining the therapeutic benefit of L-Dopa in PD.

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Author contributions

EB, EBG and POF designed research; ME, MFB, BD, ET, MB, ED, CG, EBG, QL and AP performed research. ME, MFB, ET, EBG, AP, EB and POF analyzed data. ME, MFB, EB and POF wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest

Figure legends

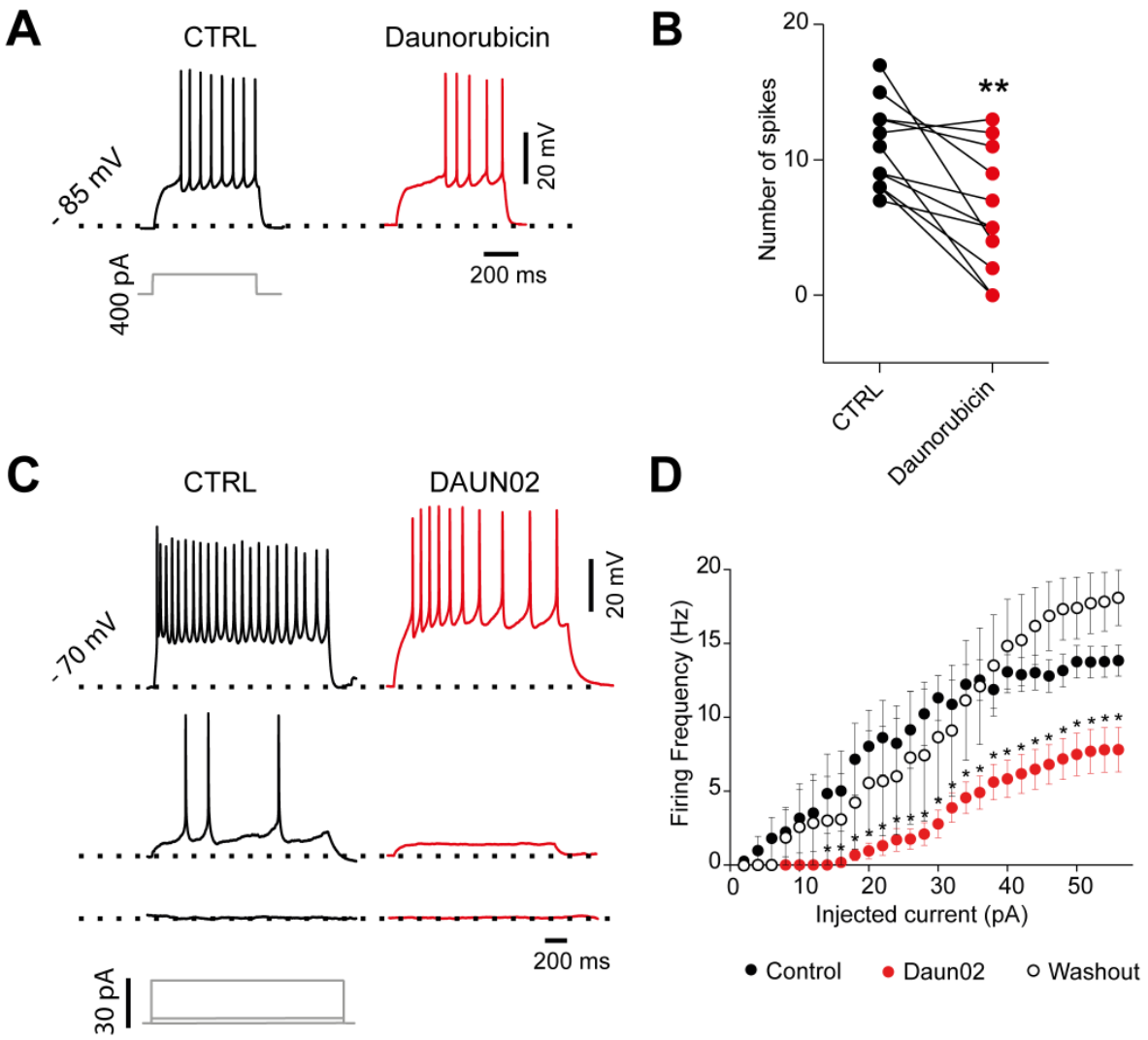

Figure 1: *Daunorubicin and Daun02 compounds induce neuronal inactivation in brain slices and striatal neuron cultures*. (**A**) Typical membrane responses of a striatal neuron to current injection (400 pA; 600 ms) before (dark trace) and after (red trace) bath application of daunorubicin in a corticostriatal slice. (**B**) Plot summarizing a decrease in the number of spikes induced by intracellular current injection after bath application of daunorubicin (1μM; 10min before recording) (** p<0.01). (**C**) Representative traces of current–clamp recordings in control conditions (dark traces) and after Daun02 incubation (2hr; 9mM; red traces) from medium spiny neurons in culture. (**D**) Plot of firing frequency as a function of the injected current in control condition (black, n = 7 cells) and after Daun02 incubation (2hr; 9mM; red; n = 22 cells). Note the decrease in striatal neuron excitability after Daun02 incubation (* p<0.05).

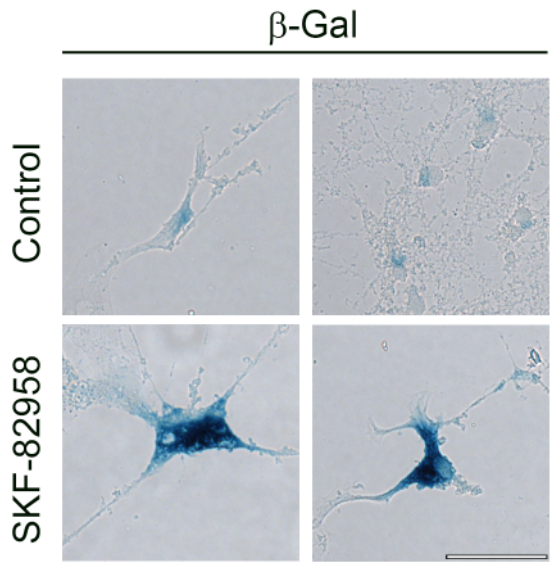

Figure 2: *Characterization of FosB-LacZ lentivirus in 15-day rat striatal cultured neurons*. A chimeric LacZ reporter lentivirus including the 5'-flanking region of the rat *FosB/ΔFosB* gene was transduced into rat striatal neurons. Cells were then exposed to 10μM of the full D1R agonist SKF-82958 for 1h. SKF-82958-induced neuronal activity induces β-galactosidase expression (blue-labeled nuclei) in neurons that express *FosB/ΔFosB*. Scale bar, 50μm.

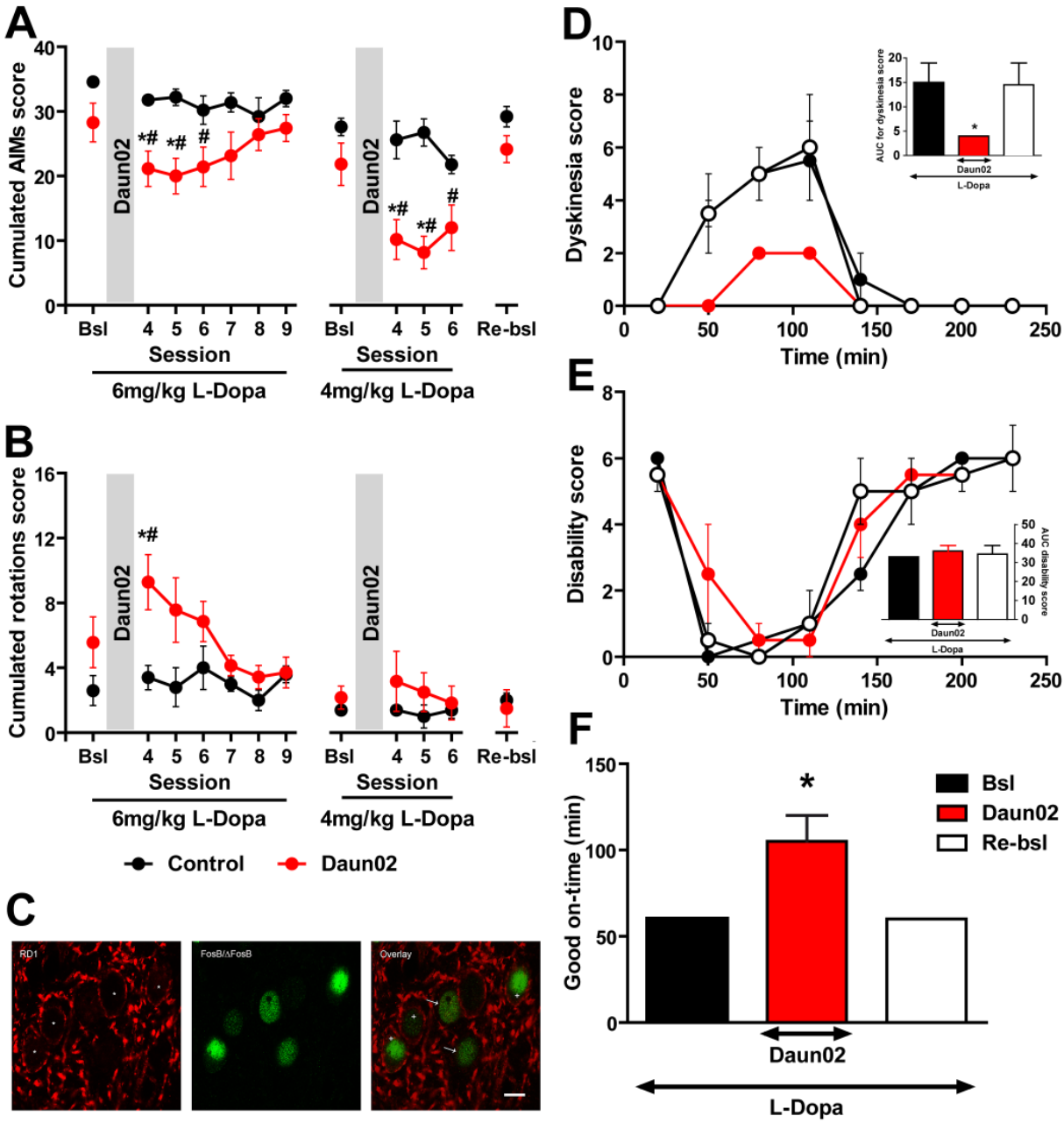

Figure 3: FosB/*ΔFosB expressing neurons inactivation reduces LID expression and spares L-Dopa beneficial effect* (**A**) cumulated AIMs scores in rats under 6mg/kg and 4mg/kg L-Dopa (ip) before and after daun02 (* p<0.05 from control; # p<0.05 from baseline); (**B**) cumulated rotation scores in rats under 6mg/kg and 4mg/kg L-Dopa (ip) before and after daun02 (* p<0.05 from control; # p<0.05 from baseline); (**C**) *receptor identity of ΔFosB-immunopositive cells*: ΔFosB accumulation occurs in both D1-positive (crosses) and -negative neurons (arrows). Asterisks indicate D1-positive cells. Scale bar 10μm. (**D**) reduction of dyskinesia scores in monkeys (**E**) without disability score impairments and (**F**) increased good on-time, (*p<0.05 from before daun02). X-gal staining revealed that 18.3 % of the striatum was transduced by the construct as previously observed with other vectors (13). Bsl: baseline; Re-Bsl: re-baselining session; AUC: Area under the curve. Mean ± SEM.

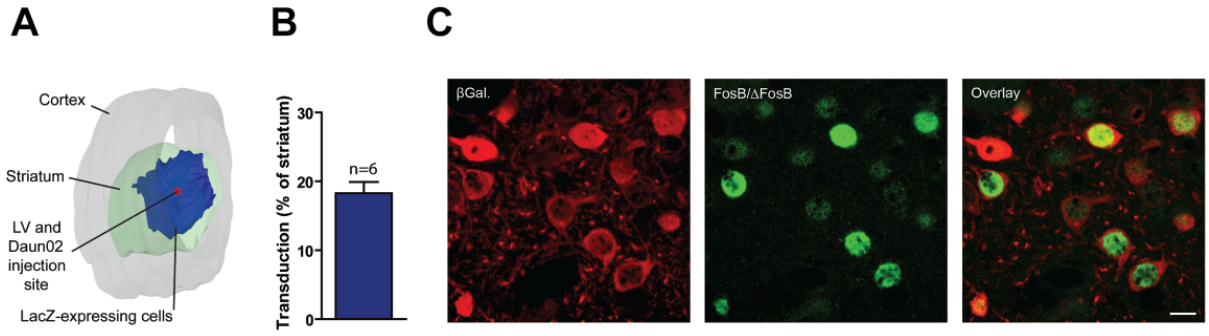

Figure 4: *LacZ expression in the striatum following stereotaxic injection of a lentivirus expressing* β-galactosidase. (**A**) Three-dimensional reconstruction of the transduction volume in the rat striatum; LV: lentivirus; (**B**) Lentiviral infection volume expressed as a percentage of the striatal volume. (**C**) Expression of β-galactosidase in FosB/ΔFosB-immunopositive cells. Scale bar 10μm.

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3. Publication 3: Inhibiting Lateral Habenula improves L-Dopa induced dyskinesia

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The systematic search of brain nuclei putatively involved in LID (publication 1) shed light, notably, upon the Lateral Habenula (LHb) which displayed an overexpression of 3 IEGs: ΔFosB, ARC and Zif268. Interestingly, in the early 90's, 2-deoxyglucose (2-DG) seminal studies showed that LHb stood up among several structures as a strongly affected non-basal ganglia nucleus displaying a dramatic increase in 2-DG accumulation in parkinsonism. We thus hypothesized that LHb might be involved in LID pathophysiology. In the present study, we demonstrate that LHb displayed a LID-related pathological activity at different functional levels including metabolic, electrophysiological and ΔFosB-related transcriptional readouts. Altogether, those data demonstrate that LHb neuronal activity in response to L-Dopa is related to LID manifestation. Then, the Daun02-driven inactivation of LHb ΔFosB-expressing neurons both alleviates LID severity and enhances the L-Dopa antiparkinsonian action, indicating an involvement of LHb both in LID expression and in the antiparkinsonian effect of L-Dopa. Taken altogether our results highlight a key role of LHb in the genesis of dyskinesia manifestation outside of the basal ganglia.

Inhibiting Lateral Habenula improves L-Dopa induced dyskinesia

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Abstract

A systematic search of brain nuclei putatively involved in L-3,4-dihydroxyphenylalanine (L-Dopa)-induced dyskinesia (LID), the debilitating side-effects of chronic dopamine replacement therapy in Parkinson's disease (PD), shed light, notably, upon the lateral habenula (LHb), which displayed an overexpression of the ΔFosB, ARC and Zif-268 immediate-early genes only in rats experiencing abnormal involuntary movements (AIMs), the rodent analog of LID. We thus hypothesized that LHb might play a role in LID. LHb was first found to be metabolically modified in dyskinetic monkeys using the 2-deoxyglucose uptake technique. Furthermore, LHb neuronal firing frequency is significantly increased only in ON L-dopa dyskinetic 6-hydroxydopamine (6-OHDA)-lesioned rats. Altogether, those data suggested that increased LHb neuronal activity in response to L-dopa is related to AIMs manifestation. Therefore, to mechanistically test if LHb neuronal activity might affect AIM severity, we targeted ΔFosB-expressing LHb neurons using Daun02-inactivation. Following induction of AIMs, 6-OHDA rats were injected with Daun02 in the LHb previously transfected with ß-galactosidase under control of the FosB promoter. Three days after Daun02 administration, animals were tested daily with L-Dopa to assess LID and L-Dopa-induced rotations. Inactivation of ΔFosB-expressing neurons significantly reduced AIM severity and also increased rotations. Interestingly, the dopaminergic D1 receptor (D1R) was overexpressed only on the lesioned side of dyskinetic rats in LHb and co-localized with ΔFosB, suggesting a D1R-mediated mechanism supporting the LHb involvement in AIMs. This study highlights the role of LHb in LID, offering a new target to innovative treatments of LID.

Introduction

Chronic treatment of Parkinson's disease (PD) patients with the dopamine precursor L-3,4-dihydroxyphenylalanine (L-Dopa) induces the development of adverse fluctuations in motor response and involuntary movements, known as L-dopa-induced dyskinesia (LID) (1, 2). The motor nature of these manifestations first led to investigating the abnormalities of neuronal function in the cortico–basal ganglia–thalamocortical motor circuits (for review, see (3-5)). Subsequent investigations using metabolic mapping unravelled that non-motor domains of the basal ganglia and beyond play also a role in these manifestations (6).

Recently, a systematic search of brain nuclei putatively involved in LID characterized ΔFosB, ARC, FRA2 and Zif268 immediate-early genes expression patterns, a class of genes rapidly transcribed in response to an external stimulus such as stimulation of the dopamine D1 receptor (D1R) (7-10). Such approach shed light notably upon structures located outside the basal ganglia. Among those, the lateral habenula (LHb) retained our attention as LHb displayed an overexpression of ΔFosB, ARC and Zif268 (9). Interestingly, Mitchell et al. showed in their 2-deoxyglucose (2-DG) seminal studies (11, 12) that, besides the now classic 2-DG uptake pattern in the basal ganglia (13, 14), LHb stood up among several structures as a strongly affected non-basal ganglia nucleus, showing dramatic increase in 2-DG accumulation in parkinsonism. We therefore postulated that LHb might play a role in LID manifestation.

In this study, we analysed the LHb 2-DG accumulation in dyskinetic MPTP-treated macaques compared to normal, parkinsonian and L-Dopa-treated parkinsonian ones, as well as LHb single-unit electrophysiological activity in ON L-dopa dyskinetic 6-hydroxydopamine (6-OHDA)-lesioned rats compared to OFF L-dopa 6-OHDA-lesioned-rats, vehicle-treated 6-OHDA rats and sham-operated rats. Finally, to test the hypothesis that the altered firing activity of LHb neurons participates to LID generation, we used FosB as a molecular marker of LID to selectively express ß-galactosidase in FosB/ΔFosB-expressing neurons and assessed the role of these ΔFosB-expressing neurons in the rat model of LID in PD (9, 15, 16) by inhibiting their electrical activity using Daun02-inactivation (17-21).

Material and Methods

Study approval

Experiments on rats were performed in accordance with the European Union directive of September 22, 2010 (2010/63/EU) on the protection of animals used for scientific purposes. The Institutional Animal Care and Use Committee of Bordeaux (CE50) approved the present experiments under the license number 5012099-A.

Experiments on primate tissues were conducted on a previously characterized brain bank (6, 13, 22, 23) collected in 1999. Experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) for care of laboratory animals. No further primate was killed for the present experiments.

2-Deoxyglucose (2-DG) procedure.

Eighteen female Macaca fascicularis monkeys (Shared Animal Health, Beijing, China) were housed in individual primate cages under controlled conditions of humidity (50 A 5%), temperature (24°C), and light (12 h light/dark cycles); food and water were available ad libitum, and animal care was supervised by veterinarians (6). Animal population corresponds to non-human primates used in the following studies (6, 13, 22, 24). 5 animals were kept as untreated-controls (6). The remaining 13 parkinsonian animals received daily MPTP (0.2 mg/kg, i.v., Sigma, St Louis, MO) according to our previously published protocol (22, 25, 26). Following stabilization of the MPTP-induced syndrome, 8 animals received twice daily 20 mg/kg of L-DOPA p.o. for 6-8 months (Modopar; Roche, Welwyn Garden City, UK; L-dopa/carbidopa ratio, 4:1). 4 monkeys displayed dyskinesia while 4 did not (6). The parkinsonian condition was assessed on a parkinsonian monkey rating scale using videotape recordings of monkeys (24, 27). A score of 0 corresponds to a normal animal, and a score of >6 corresponds to a parkinsonian animal (24). The severity of dyskinesia was rated using the dyskinesia disability scale (28, 29): 0, dyskinesia absent; 1, mild, fleeting, and rare dyskinetic postures and movements; 2, moderate, more prominent abnormal movements, but not interfering significantly with normal behavior; 3, marked, frequent, and, at times, continuous dyskinesia intruding on the normal repertoire of activity; or, 4, severe, virtually continuous dyskinetic activity, disabling to the animal and replacing normal behavior.

On the day they were killed, monkeys were given an intravenous injection of 1 mCi/kg [^{3}H] 2-DG (specific activity, 50 Ci/mmol, 185 GBq/mmol; Interchim, Grenoble, France) in sterile saline as described previously (6, 12, 13). After 45 min, all animals were killed by sodium pentobarbital overdose (150 mg/kg, i.v.). L-Dopa-treated animals received L-dopa 15 min

before 2-DG. Brains were quickly removed, immediately frozen in isopentane (-45°C) and stored at -80°C. Tissue was sectioned at 20 μm in a cryostat at -17°C and thaw-mounted onto gelatin-coated slides. Once freeze-dried (-60°C; 40.10^{-3} atmospheres) for 2 h, both serial sections and autoradiographic methylmethacrylate standards (Amersham Biosciences, Uppsala, Sweden) were exposed to ^{3}H-Hyperfilm (Amersham Biosciences) for 2 months at -30°C, developed in D-19 developer (Eastman Kodak, Rochester, NY), and fixed in Kodak Unifix. Densitometric analysis of autoradiographs was performed using an image analysis system (Visioscan version 4.12; Biocom, Les Ulis, France) as described previously (6, 13). An examiner blind with regard to the experimental condition analyzed two sections of LHb per animal. Optical densities were averaged in each animal and converted to the amount of radioactivity bound in comparison with the standards. Mean bound radioactivity and SEM were then calculated for each group.

Electrophysiological single-unit experiments

Adult Sprague-Dawley male rats (Charles River Laboratories, Lyon, France), weighing 175-200g at the beginning of the experiment, were used. They were housed under standard laboratory conditions in a 12-hour light/12-hour dark cycle with free access to food and water. On Day 0, unilateral injection of 6-OHDA (2.5 μl at 3μg/μl) was performed in the right medial forebrain bundle (AP=-3.7mm; ML=+1.6mm; DV=-8mm relative to Bregma (30)), in rats treated 30 minutes before with citalopram (1mg/kg i.p.) and desipramine hydrochloride (20mg/kg i.p.) according to previously published procedures (9, 15, 16, 31, 32). 15 rats displaying an impaired stepping test (9, 15, 31, 33, 34) assessed on days 18 to 20 and a loss of tyrosine hydroxylase-immunopositive fibers in the striatum greater than 95% (3, 4) were considered as lesioned and were retained for experiments. 7 rats were kept as 6-OHDA-lesioned rats. From day 21 onwards, 8 rats received once daily an i.p. injection of a combined dose of benserazide (15mg/kg) and L-DOPA (6mg/kg) for 10 days (ON and OFF L-dopa dyskinetic 6-OHDA-lesioned). At the 31th day, baseline abnormal involuntary movements (AIMs) score was assessed. The 4 AIMs categories (limb, axial, orolingual, and locomotive) were scored using a validated rating scale (35, 36) for 1 minute every 20 minutes for 2 hours (total 4 observations; maximal score for each observation, 16; maximal total score per session, 64) performed by a trained investigator as previously described (9, 15, 31, 32, 37-39). Electrophysiological recordings were performed in the right LHb (AP= -3.5 to -4mm; ML= +0.5 to 1 mm; DV= -4.2 to -5 mm, (30)) in anesthetized ON L-dopa dyskinetic 6-OHDA-

lesioned rats with repeated L-Dopa injection each 90 minutes (n=8), OFF L-dopa 6-OHDA-lesioned-rats (i.e. dyskinetic rats which did not receive L-Dopa on the recording day, n=8), vehicle-treated 6-OHDA rats (n=7) and sham-operated rats (n=10). Anaesthesia was induced with 3% isofuran and maintained with urethane (1.25 g/kg, i.p.) and supplemental doses of ketamine (30 mg/kg, i.p.) and xylazine (3 mg/kg, i.p.), as described previously (40-42). Extracellular recordings of single-unit activity in the LHb were made using glass electrodes (10–20 MΩ in situ; tip diameter ~1.2 μm) containing 0.5 M NaCl solution and neurobiotin (2% w/v; Vector Laboratories, USA). Recording signals were performed as previously described (43). Briefly, it was amplified 10-fold with an Axoclamp2B (Molecular Devices, USA) in the bridge mode versus a reference electrode implanted in the neck skin. It was further amplified 100-fold with differential AC amplifier (model 1700; A-M Systems, USA) and divided in two channels. One was used for spike recording (300-10000 Hz) and the other for local field potential acquisition (0.1-10000 Hz). Then, extracellular potential was digitalized using the Micro1401-3 and analysed with Spike2 software (Cambridge Electronic Design, UK). Following electrophysiological recordings, single neurons were juxtacellularly labelled with neurobiotin as previously described (41, 42, 44, 45).

All electrophysiological recordings were performed in the slow-wave activity brain state. Brain state was qualitatively assessed for each rat through an electrocorticogram (ECoG) recorded via a 1-mm-diameter screw juxtaposed to the dura mater above the right motor cortex M1 (AP=+3.5 mm; L=+3.5 mm, (30)), and referenced with another screw inserted in the skull above the right cerebellar. Raw ECoG was bandpass filtered (0.1–5000 Hz) and amplified 1000-fold (model 1700; A-M Systems, USA) before it acquisition by Micro1401-3 and Spike2 software. For all electrophysiological recordings, the sampling rate was fixed at 20 kHz.

At the end of the recording, rats were perfused transcardially with 0.9% NaCl followed by ice-cold 4% formaldehyde in PBS. Brains were removed, postfixed overnight in the same fixative (4°C), then cryoprotected for 48h at 4°C in 20% PBS-sucrose. Brains were frozen in isopentane at -45°C and stored at -80°C until sectioning following by neurobiotin staining as described previously (41, 42, 44, 45).

Stereological data analysis for correlation

The number of ΔFosB-immunopositive cells was obtained as previously described (9) applying the optical fractionator (9, 34, 46, 47) unbiased stereological method using a Leica DM6000B microscope with Mercator Pro software (ExploraNova, version 7.9.8). Immuno-

labelled cells were counted by a blind investigator on every 6th section previously used (9) with stereological parameters adapted to LHb (Counting frames: 60x60µm, Spacing: 100x100µm, number of sections : 3). Animal population corresponds to rats used from our precedent published study: dyskinetic 6-OHDA-lesioned rats (n=5) and non-dyskinetic 6-OHDA lesioned rats (n=5) (9).

Daun02/ß-galactosidase inactivation method

12 dyskinetic L-Dopa-treated 6-OHDA-lesioned rats were obtained as described above except that at the same time of 6-OHDA injection, all the animals were injected with 2µl of a lentiviral vector expressing LacZ (coding for ß-galactosidase) under control of a FosB promoter with a final titer of $1.18x10^9$ infectious particles/ml as previously used (21) in LHb (AP=-3.48mm; ML=+0.65 mm; DV=-4.4mm). Guide cannulas were implanted as previously described (15, 21) (AP=-3.48mm; ML=+0.65mm; DV=-4.2mm) and cemented to the skull for subsequent Daun02 injections (21). 31 days post-6-OHDA and lentiviral injections, baseline AIMs score was assessed as described above. On the 32th day, animals received a 6mg/kg L-dopa injection 1h before a 1µL Daun02 injection (4 µg/µL in 5% DMSO, 5% Tween-80 in PBS at 0.5 µl/min) in LHb under light isoflurane anesthesia before being placed in their home cage for 3 days as described (18, 19, 21). From the 3rd day after Daun 02 injection, all rats received a daily 6mg/kg L-Dopa injection and AIMs were scored (21). To ensure reversibility of Daun02-induced inactivation, a control solution (5% DMSO, 5% Tween-80 in PBS at 0.5 µl/min) was injected in the same animals 6 days after Daun02 injection and AIMs were evaluated.

At the end of the Daun02 experiment, 1 hour after the last L-DOPA injection, i.e. at the peak of behavioural effect, rats were deeply anesthetized with chloral hydrate (400mg/kg, i.p., VWR) and perfused transcardially with 0.9% NaCl followed by ice-cold 4% formaldehyde in PBS. Brains were removed, postfixed overnight in the same fixative (4°C), then cryoprotected for 48h at 4°C in 20% PBS-sucrose. Brains were frozen in isopentane at -45°C and stored at -80°C until sectioning.

Histological data analysis

50µm-thick cryostat-cut coronal rat brain sections were collected and processed for tyrosine hydroxylase (MAB318, Milipore), ΔFosB (sc-48, Santa-Cruz), D1R (D2944, Sigma) as previously described (9, 21, 46), and ß-galactosidase (AB1211-5MG, Millipore) immunohistochemistry (21)

Data Analysis

2-DG and electrophysiological neuronal frequency data were analyzed using one-way analysis of variance (ANOVA) used to estimate overall significance, followed by *post hoc* t tests corrected for multiple comparisons by Bonferroni's method (48). Electrophysiological neuronal pattern were analyzed using chi-squared test (27). Electrophysiological analyses were conducted on neurons that present at least 500 spikes during epochs of cortical slow-wave activity selected as previously described (45, 49). Firing rate was calculated using Neuroexplorer (Nex Technologies, USA) while overall neuron (27, 50) firing patterns were analyzed using density histogram method (51) as previously described (52, 53). Behavioural Data were analyzed with wilocoxon-signed rank t-test (54). All data are presented as mean ± SEM with a threshold for statistical significance at $p<0.05$. Correlations between LID severity and ΔFosB immuno-positive counts were performed using Spearman correlation (9).

Results

LID involve metabolic, electrophysiological and transcriptional alterations in LHb

2-DG uptake was measured in LHb to assess the metabolic activity induced by LID manifestation in monkeys (6, 13). Interestingly, 2-DG accumulation in LHb ($F_{(3,14)} = 35.71$, $p<0.001$) significantly decreases in dyskinetic monkeys compared to non-dyskinetic ($p<0.05$) but also compared to MPTP-lesioned ($p<0.001$) and control monkeys ($p<0.05$) **(Figure 1A)**. No significant modification was found between control and non-dyskinetic monkeys while 2-DG uptake is impressively enhanced in MPTP-lesioned monkeys compared to dyskinetic, non-dyskinetic, and control monkeys ($p<0.001$ vs. all) **(Figure 1A)** in accordance with the original report (11). Those data suggest that the parkinsonism-induced enhancement in the activity of LHb inputs is dramatically decreased in the dyskinetic animals. Such decrease in activity inputs is further visible when comparing dyskinetic and non-dyskinetic animals.

Following 2-DG experiments in monkey, we analysed the LHb neuronal discharge frequency and pattern in the 6-OHDA-lesioned rat model of PD and LID **(Figure 1B**). While dyskinetic 6-OHDA animals recorded when OFF L-dopa did show a LHb firing frequency comparable to that of drug naïve 6-OHDA and sham-operated rats **(Figure 1C)**, the ON L-dopa dyskinetic 6-OHDA-lesioned rats displayed a dramatic increase in firing frequency significantly distinguishing them from all other groups ($F_{(3,88)} = 10.30$, $p<0.05$); **Figure 1C)**. Regarding LHb neuronal pattern, both ON L-Dopa dyskinetic 6-OHDA-lesioned rats and OFF L-Dopa

6-OHDA-lesioned rats display a significant difference compared to sham-operated rats ($p<0.05$) **(Figure 1D)**. Those data further suggest that dyskinetic manifestations are associated with pathological changes both in the firing rate and patterns of LHb neurons.

Finally, we established that the ΔFosB-transcriptional response in LHb induced by chronic L-dopa in 6-OHDA lesioned rats (9) linearly correlated with the severity of abnormal involuntary movements (AIMs) (R^2: 0,91, $p<0.001$) **(Figure 1E)**.

Altogether the data suggested that LID-related changes in LHb metabolic, transcriptional and electrophysiological activities allow distinguishing the dyskinetic animals from the non-dyskinetic ones, making the LHb a putative key relay in the genesis of dyskinesia manifestation.

Inhibition of Habenular ΔFosB-expressing neurons alleviates LID

To directly assess the casual role of LHb upon AIM severity, in the rodent analog of dyskinesia, we inactivated the electrical activity of ΔFosB-expressing LHb neurons using the selective Daun02/β-galactosidase inactivation method. This method consists into the local administration of the prodrug Daun02 converted into daunorubicin by ß-galactosidase, readily expressed in mammalian cells previously transduced with the E. coli LacZ gene under the control of a cell-specific promoter (17-19). A FosB-LacZ lentivirus, therefore expressing ß-galactosidase only in FosB/ΔFosB-expressing neurons (21), was injected *in vivo* in LHb of 6-OHDA-lesioned rats chronically treated with L-Dopa (5, 9, 15, 16). After the establishment of stable AIMs, a single intra-LHb administration of Daun02 significantly decreased AIMs compared to baseline score ($p<0.05$; **Figure 2A**). AIMs reduction lasted 3 days compared with baseline score (21%, 24% and 15% respectively; $p<0.05$ for all; **Figure 2A**) in keeping with previous demonstration of Daun02-mediated behavioral span (19). After a return to baseline AIMs score, a control solution, (vehicle without Daun-02), was injected in LHb of the same rats. No significant difference in AIMs score was found between vehicle-treated rats and baseline scores while Daun-02-inactivation induced a significant decrease in AIMs score compared to vehicle injection for 3 days ($p<0.05$ for all; **Figure 2A**). Moreover, Daun02 increased rotational behavior, an index of the anti-parkinsonian effect of L-Dopa (5, 36) also associated to LID, compared with both baseline and control-treated rats (69%; $p<0.05$ for all; **Figure 2B**).

Increased habenular D1R expression colocalizes with ΔFosB *expression*

Immunofluorescence assay revealed an increased expression of D1R only on the lesioned side of dyskinetic 6-OHDA-Lesioned rats compared to 6-OHDA-lesioned and sham-operated ones **(Figure 2C)** suggesting that chronic L-dopa increases D1R expression in LHb neurons. In addition, double immunofluorescence of D1R/ΔFosB uncovers a co-localization of ΔFosB and D1R in LHb of the lesioned side of dyskinetic rats **(Figure 2D)** suggesting that, as in other dopaminoceptive areas, ΔFosB rise is induced by D1R stimulation. ß-galactosidase immunofluorescence confirmed an expression of the FosB/LacZ lentivirus restricted to LHb **(Figure 2E)**, ascertaining the LHb nature of the observed behavioral manifestations.

Discussion

LID have been associated with both presynaptic and postsynaptic mechanisms at the striatal level in the basal ganglia (3-5). In this study, we report that LHb is functionally and behaviourally involved in LID pathophysiology in accordance with growing evidences supporting the involvement of outside basal ganglia structures in LID (6, 9, 55, 56). First, we revealed a LID-related pathological activity of LHb at different functional levels including metabolic, transcriptional and electrophysiological readouts, indicating that increased LHb activity in response to L-Dopa treatment is associated with LID expression. Then, selective inactivation of ΔFosB-expressing habenular neurons both alleviates LID severity and enhances L-dopa antiparkinsonian action, suggesting an involvement of LHb both in LID severity and in the antiparkinsonian effect of L-Dopa therapy. Taken altogether our results highlight a key role of LHb in the genesis of dyskinesia manifestation outside of the basal ganglia.

The amount of 2-DG uptake correlates directly with the magnitude of the mean synaptic activity and is therefore considered to be a measure of the global afferent activity of a structure (57-59). However, this technique does not allow distinction between a modification in excitatory and in inhibitory afferent activity (60). Furthermore, 2-DG uptake reflects the activity of all cellular elements in the region of interest, i.e. perikarya, dendrites, axonal fibres and glia. Despite these limitations, a classic example of dissociated 2-DG uptake and electrical activity is given by the subthalamic nucleus (STN) in PD that becomes hyperactive and bursty while displaying a decreased 2-DG accumulation (6, 11-13, 61). In addition, disinhibition of STN neurons by local injection of bicuculline, a GABA antagonist, increases the firing rate of STN neurons as well as the firing rate and 2-DG uptake in the globus pallidus, the entopeduncular nucleus and the SNr in the rat (60). The opposite can be observed

when locally injecting muscimol, a GABA agonist, suggesting that inhibition of STN neurons decreases the mean afferent activity and firing rate in its target nuclei (60).

In our study, the metabolic (2-DG) and electrophysiological endpoints of the LHb are dissociated as well leading to posit about the role of LHb in pathophysiology of LID.

LHb is mainly innervated by the output structures of the basal ganglia while minor afferents arise from the ventral tegmental area (VTA), lateral hypothalamus and lateral preoptic area (62-67). LHb indeed receives inhibitory afferents from the ventral pallidum (VP) (63, 64), but also receives excitatory afferents from the border cells of the internal part of the globus pallidus (GPi) (62, 63). The elevated LHb 2-DG uptake in parkinsonian monkeys (compared to control and L-Dopa treated ones) therefore suggests an increase in afferant activity converging towards the LHb, in keeping with initial studies (11, 12). The GPi overactivity in PD (52, 68-72) was thought to be responsible from such increase. Contrary to most GPi neurons, LHb-innervating excitatory GPi border cells (62, 63) show a decreased firing rate in parkinsonism (73), enlightening the lack of difference in LHb neuronal discharge frequency between control and 6-OHDA-lesioned rats. In presence of LID, however, those GPi border cells present a significant increase in firing rate associated with a pattern modification compared to parkinsonism and control states (73). Consequently, habenular neurons firing rate, driven by border cells input, is increased specifically in dyskinetic rats.

LHb is primarily seen as a relay connecting the limbic system and the basal ganglia with monoaminergic centres (74). LHb projects mainly to monoaminergic brain regions including: dopaminergic areas (i.e. ventral tegmental area (VTA) and substantia nigra compacta (SNc)) serotoninergic areas (i.e. dorsal and medial raphe) and also to the cholinergic laterodorsal tegmentum (74-77). Recent evidences suggest that LHb plays a critical role in dopaminergic-related processes including drug abuse and reward (74, 77-80). Interestingly, cocaine administration increases LHb neuronal firing following D1R and D2R stimulation (79) while the specific LHb inactivation through deep brain stimulation decreases cocaine-seeking behaviour (81). Dopaminergic receptor activation through systemic apomorphine injection strongly enhances spontaneous activity of distinct habenular neuron subsets (82). Those data are reminiscent of the present results with hyperdopaminergia-induced increased activity and inhibition of LHb neurons resulting into improvement of the hyperdopaminergia-induced behaviour.

Interestingly, increased expression of D1R in LHb of dyskinetic 6-OHDA-lesioned rats ipsilateral to the lesion unravels a direct D1R-related mechanism in engaging LHb in LID pathophysiology. LID derive from sensitized D1 receptors due to chronic L-Dopa stimulation

(15, 83). Therefore, while the key role of striatal D1R in LID has been well described, ascertaining a role for extrastriatal, e.g. intra LHb, D1R may seem provocating. It however shares enough similarity with striatal involvement for being a realistic hypothesis (3-5). Indeed, as in the striatum, L-Dopa induces an overexpression of ΔFosB in LHb which (i) correlates with LID severity, (ii) co-localizes with D1R and (iii) drives, at least in part, LID expression. Fos-like IEGs are directly related to the D1R pathway both in the striatum (7) and LHb (84) as their expression is directly enhanced by specific D1R agonist. Altogether, these data suggest an involvement of D1R/ΔFosB habenular neurons in LID pathophysiology.

How LHb neurons impact LID behaviour remains however unsolved. LID pathophysiology involves post-synaptic mechanisms but also presynaptic dysfunctions with notably, the false neurotransmitter hypothesis. Exogenous L-dopa is indeed mostly uptaken by serotonergic terminals, dopamine becoming the false neurotransmitter of those serotonin neurons (85, 86). LHb is heavily projecting upon serotonin neurons of the raphe (75). While the dopaminergic areas are markedly lesioned, the serotonergic ones are relatively preserved by the neurotoxin insult (87, 88). Thus, impaired LHb output would participate to the aberrant dopamine release from serotonin terminals (87, 89-92) and hence impact LID.

In pre-clinical models, most anti-dyskinetic drugs can negatively affect the duration and/or magnitude of the therapeutic effect of L-Dopa, highlighting their lack of strict selectivity towards the underlying mechanisms of LID (93, 94). In this study, LID reduction through LHb selective inactivation was associated with a remarkable increased in L-Dopa-induced rotational behaviour. Therefore, even if the increase in rotational behaviour could be a consequence of a decrease in AIMs and vice versa, LHb should be considered as a key player in mediating the anti-parkinsonian effect of L-Dopa through specifically ΔFosB expressing neurons.

Conclusion

Our results show that LHb is involved both in LID and in the anti-parkinsonian effect of L-Dopa. LID impact metabolic, electrophysiological and transcriptional events in LHb while the inactivation of habenular neurons alleviates LID. Even if the underlying mechanisms involving LHb in LID pathophysiology are not yet completely elucidated, our data suggest that these effects should be mediated, at least in part, by D1R/ΔFosb expressing neurons. Taken altogether, our results highlight the role of LHb in LID, offering a new target to innovative treatments of LID.

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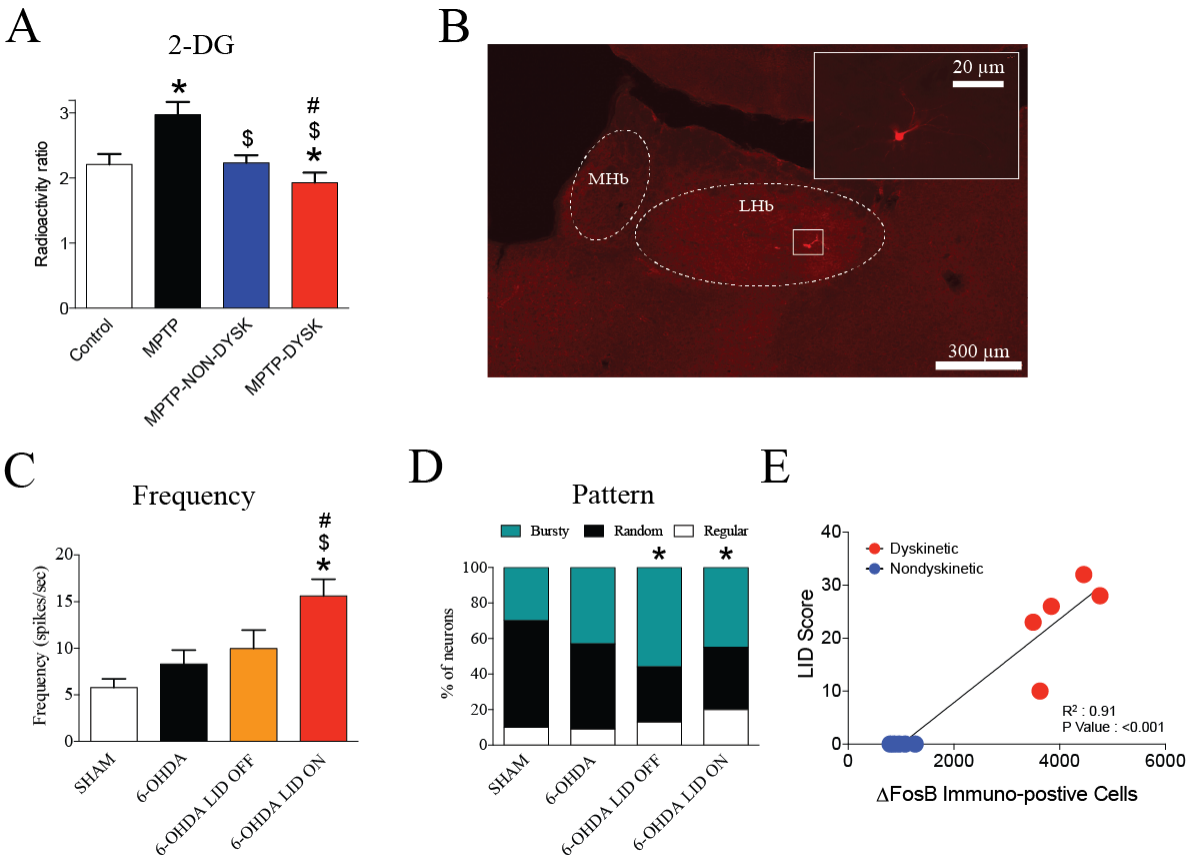

Figure 1. LID impact metabolic, electrophysiological and transcriptional responses in LHb. **A-** Densitometric analysis of 2-dexoyglucose (2-DG) accumulation in control (n=5), parkinsonian (MPTP; n=5), L-Dopa non-dyskinetic (MPTP-non-dysk; n=4) and L-Dopa dyskinetic macaque monkeys (MPTP-dysk; n=5). Data are expressed in terms of tissue equivalent ratios of the amount of radioactivity in the considered structure to that in the white matter of the same section (* $p<0.05$ from control, \$ $p<0.05$ from MPTP, # $p<0.05$ from MPTP-non-dysk). **B-** Representative example of LHb neurobiotin-injected neuron in the rat after electrophysiological recording, scale bar 300µm (with an inset magnification, scale bar 20µm); MHb = Medial Habenula, LHb = Lateral Habenula. **C-** LHb neuronal firing frequency (spike/sec) analysis between sham-operated rats (n=30 neurons), vehicle-treated 6-OHDA rats (n=21 neurons), ON L-Dopa dyskinetic 6-OHDA-lesioned rats (6-OHDA LID ON, n=20 neurons) and OFF L-Dopa 6-OHDA-lesioned-rats (6-OHDA LID OFF, n=16 neurons) (* $p<0.05$ from sham, \$ $p<0.05$ from 6-OHDA, # $p<0.05$ from 6-OHDA LID OFF). **D-** LHb neuronal firing pattern (% of neurons recorded) analysis between sham-operated rats (n=30 neurons), vehicle-treated 6-OHDA rats (n=21 neurons), ON L-Dopa dyskinetic 6-OHDA-lesioned rats (6-OHDA LID ON, n=20 neurons) and OFF L-Dopa 6-OHDA-lesioned-rats (6-OHDA LID OFF, n=16 neurons) (* $p<0.05$ from sham-operated rats). **E-** Correlation between AIM severity and number of LHb ΔFosB immuno-positive neurons (R^2: 0,91, $p<0.001$) in dyskinetic (red) and non-dyskinetic (blue) 6-OHDA-lesioned rats.

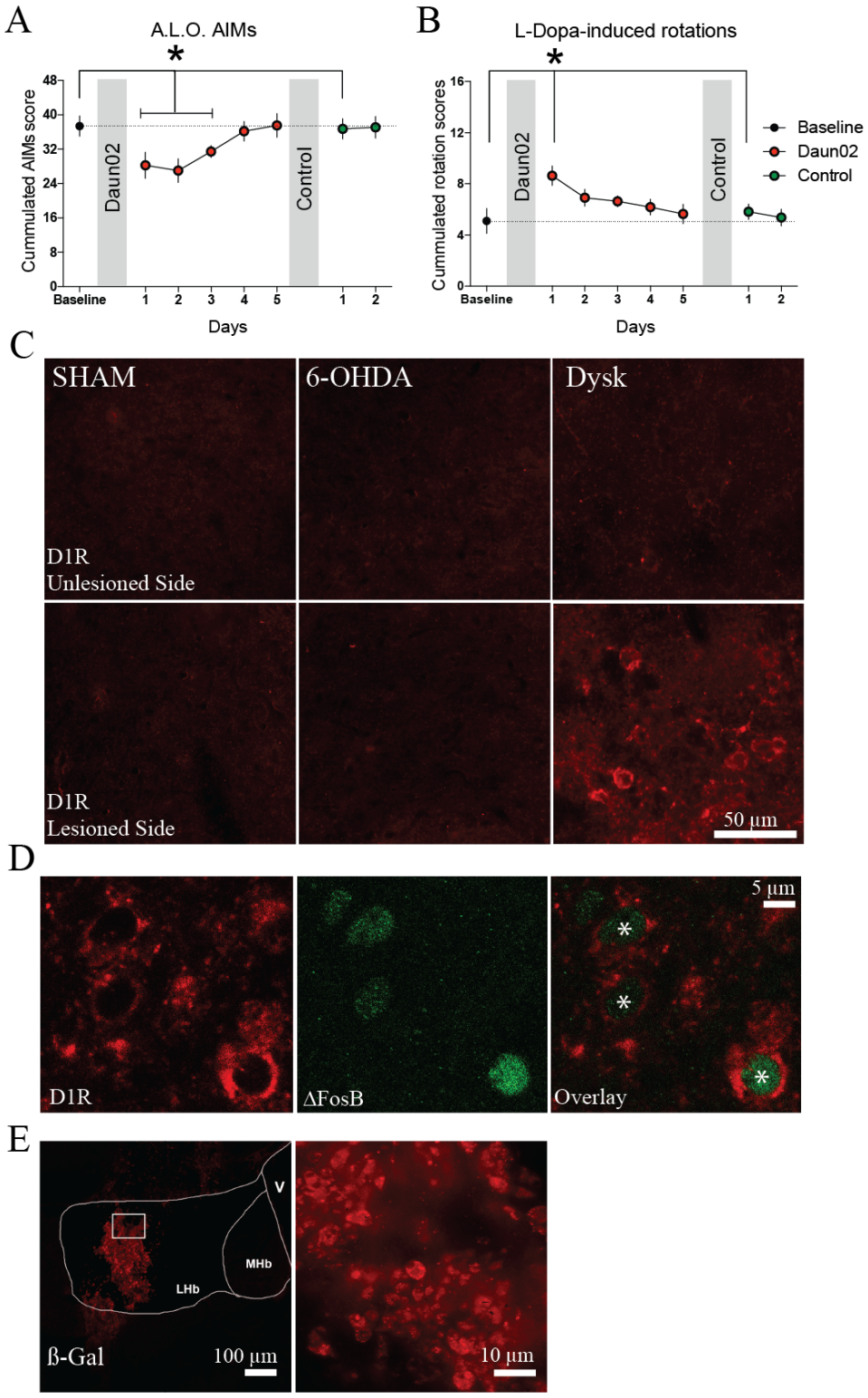

Figure 2. Inhibition of ΔFosB-expressing LHb neurons alleviates LID. **A**- Cumulated axial, limb and orofacial (A.L.O.) AIMs scores in L-Dopa-treated 6-OHDA rats (n=12) before and after Daun02 and after control solution injection (* $p<0.05$ from baseline and control). **B**- Cumulated rotation scores in L-Dopa-treated 6-OHDA rats (n=12) before and after Daun02 and after control solution injection (* $p<0.05$ from baseline and control). **C**- Representative LHb mapping of D1R expression in sham-operated (SHAM), 6-OHDA-lesioned (6-OHDA), and Daun02-injected-6-OHDA-lesioned dyskinetic rats (Dysk). Scale bar 50µm. **D**- Co-localization of D1R/ΔFosB (*) expression in LHb neurons in the Daun02-injected-6-OHDA-lesioned side of dyskinetic rats. Scale bar: 5µm **E**- Representative LHb ß-galactosidase (ß-Gal) expression in the Daun02-injected side of dyskinetic rats (scale bar: 100µm) with an inset (scale bar: 10µm). LHb = Lateral Habenula; MHb = Medial Habenula; V = Ventricule.

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4. Publication 4: Involvement of an outside basal ganglia nucleus in L-Dopa induced dyskinesia: the bed nucleus of the stria terminalis

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In preparation

In addition to the LHb, the IEG whole brain screening of dyskinetic 6-OHDA-lesioned rats (publication 1) identified the dorsolateral part of the bed nucleus of the stria terminalis (dlBST), which displayed an overexpression of the 4 IEGs: ΔFosB, ARC, Zif268 and FRA2. The reason why we targeted the dlBST among the identified nuclei is two-fold. First, a 2-deoxyglucose (2-DG) study demonstrated that a chronic L-Dopa treatment induces a decrease in 2-DG accumulation only in the BST of dyskinetic MPTP-lesioned macaques. Secondly, we found a significant correlation between dlBST ΔFosB-immuno positive cells and LID severity. Therefore, we hypothesized that dlBST might be involved in the expression of LID. In the present study, we first showed that the Daun02-driven inactivation of dlBST ΔFosB-expressing neurons alleviates LID severity in dyskinetic rats. Remarkably, we also confirmed the BST involvement in the gold standard model of LID, the dyskinetic MPTP-lesioned macaque. We then demonstrated that a dopaminergic D1 receptor (D1R) agonist increases the $GABA_A$-mediated inhibitory synaptic transmission only in the dlBST oval nucleus (ovBST) of dyskinetic-6-OHDA-lesioned rats associated with an increase in D1R expression. Altogether, our results highlight the functional involvement of another extra-striatal structure in LID both in dyskinetic rats and monkeys, offering a new target to innovative treatments of LID.

Involvement of an extra-striatal nucleus in L-Dopa induced dyskinesia: the bed nucleus of the stria terminalis

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Abstract

A whole brain search approach highlighted the dorsolateral bed nucleus of the stria terminalis (dlBST) as a putative nucleus involved in L-3,4-dihydroxyphenylalanine (L-Dopa)-induced dyskinesia (LID), the debilitating side-effects of chronic dopamine replacement therapy in Parkinson's disease (PD), which displayed an overexpression of ΔFosB, ARC, Zif268 and FRA2 only in dyskinetic rats. We thus hypothesized that dlBST could play a role in LID pathophysiology. In order to assess the causal role of the dlBST in LID, we inactivated the electrical activity of dlBST ΔFosB-expressing neurons using Daun02-inactivation. Following induction of abnormal involuntary movements (AIMs), 6-OHDA rats were injected with Daun02 in the dlBST previously transfected with ß-galactosidase under control of the FosB promoter. Three days after Daun02 administration, animals were tested daily with L-Dopa to assess LID. Inactivation of ΔFosB-expressing neurons significantly reduced AIM severity. Remarkably, as a proof of concept, we confirmed the dlBST involvement in the gold standard model of LID: the dyskinetic MPTP-treated macaque. We then unravelled a significant increase in D1 modulation of $GABA_A$-mediated inhibitory synaptic transmission only in the dlBST oval nucleus of dyskinetic rats associated to an increased in D1R expression, suggesting the involvement of a D1-related mechanism engaging the dlBST in LID. The present study highlights the role of dlBST in LID, both in rodent and non-human primate, offering a new target to innovative treatments of LID.

Introduction

The gold standard treatment for Parkinson's disease (PD) remains the dopamine precursor L-3,4-dihydroxyphenylalanine (L-Dopa). Long-term L-Dopa treatment systematically leads to abnormal involuntary movements (AIMs) called L-DOPA-induced dyskinesia (LID) [1, 2]. From the 90's to nowadays, growing evidences suggest that the mechanisms underlying PD and LID pathophysiology do not involve only motor regions but also associative and limbic domains of the basal ganglia and beyond [3-7], notably the bed nucleus of the stria terminalis (BST) [3].

Recently, a whole brain search approach highlighted the dorsolateral (dl) BST, which displayed an overexpression of 4 independent IEGs: ΔFosB, ARC, Zif268 and FRA2 [8] only in dyskinetic 6-OHDA-lesioned rats. The dlBST is composed of 2 nuclei, the oval (ovBST) and juxta (jxBST) which both showed a significant correlation between ΔFosB or FRA2 expression and LID severity [8]. Altogether, these evidences led us to hypothesize that the dlBST could be actively involved in LID manifestations.

Interestingly, striatal down-regulation of FosB expression or electrical inhibition of FosB/ΔFosB-expressing neurons decrease LID severity both in rats and non-human primates [9-11], demonstrating that FosB/ΔFosB is not only a marker of LID but that inhibiting its function or the neurons expressing it functionally impact AIMs. Therefore, to assess the role of the dlBST in LID pathophysiology, we used the FosB promoter to selectively drive the expression of the ß-galactosidase in FosB/ΔFosB-expressing neurons. We assessed the role of these ΔFosB-expressing neurons in the rat and non-human primate models of LID in PD [8, 11-13] by inhibiting their electrical activity with the Daun02-inactivation method [14-17, 11]. Then, in order to identify the neuronal mechanisms involving the dlBST in LID pathophysiology, we analysed the excitatory and inhibitory synaptic transmission both in the ovBST and jxBST of dyskinetic rats by quantifying (i) the AMPA/NDMA ratio, (ii) the AMPA-mediated excitatory transmission and (iii) the GABAa-mediated inhibitory transmission.

Material and Methods

Study approval

Experiments on rats were performed in accordance with the European Union directive of September 22, 2010 (2010/63/EU) on the protection of animals used for scientific purposes. Experiments on non-human primates were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) for care of laboratory animals. The Institutional Animal Care and Use Committee of Bordeaux (CE50) approved the present experiments under the license number 5012099-A.

Daun02/ß-galactosidase inactivation method

Rat experiments

Adult Sprague-Dawley male rats (Charles River Laboratories, Lyon, France), weighing 175-200g at the beginning of the experiment, were used. They were housed under standard laboratory conditions in a 12-hour light/12-hour dark cycle with free access to food and water. On Day 0, unilateral injection of 6-OHDA (2.5 µl at 3µg/µl) was performed in the right medial forebrain bundle (AP=-3.7mm; ML=+1.6mm; DV=-8mm relative to Bregma [18]), in rats treated 30 minutes before with citalopram (1mg/kg i.p.) and desipramine hydrochloride (20mg/kg i.p.) according to previously published procedures [8, 12, 13, 19, 20].

At the same time, all the animals were injected with 250nl of a lentiviral vector expressing LacZ (coding for ß-galactosidase) under control of a FosB promoter with a final titer of $1.18x10^9$ infectious particles/ml as previously used [11] in the dlBST (AP=-0.4mm; ML=+1.8mm; DV=-5.6/-7.2mm). All lentiviral injections were performed following electrophysiological recordings of the dlBST as we previously performed [21]. Stimulation and recording electrodes were inserted into the insular cortex (INS Cx; AP=-0.2mm; ML=+5.8 mm; DV=-4.4mm) or the Ov/JxBST (AP=-0.4mm; ML=+1.8mm; DV=-5.6/-7.2mm), respectively. Bipolar electrical stimulation of the INS Cx was conducted with a concentric electrode (Phymep) and a stimulus isolator (500µs, 0.2-2 mA; Digitimer). Baseline was recorded for 10 min (2x100 pulses; 0.5Hz). Ov/jxBST recordings were performed using a glass micropipette (tip diameter, 1-2 µm; 10-15-MΩ) filled with a 2 % sky blue pontamine solution in 0.5M sodium acetate. The extracellular potential was recorded with an Axoclamp-2B amplifier and filtered (300 Hz/0.5Kz) [21]. Single neuron spikes were collected online (CED 1401, SPIKE2; Cambridge Electronic Design). During electrical stimulation of the INS Cx, cumulative peristimulus histograms (PSTHs, 5ms bin width) of ov/jxBST activity were generated for each neuron recorded. Then, guide cannulas were implanted as previously

described [11, 12] (AP=-0.4mm; ML=+1.8mm; DV=-5.6/-7.2mm) and cemented to the skull for subsequent Daun02 injections.

Rats displaying an impaired stepping test [8, 12, 19, 22, 23] assessed on days 18 to 20 and a loss of tyrosine hydroxylase-immunopositive fibers in the striatum greater than 95% [24, 25] were considered as lesioned and were retained for experiments. From day 21 onwards, rats received once daily an i.p. injection of a combined dose of benserazide (15mg/kg) and L-DOPA (6mg/kg) for 10 days. At the 31th day post-6-OHDA and FosB-LacZ lentiviral injections, the baseline abnormal involuntary movements (AIMs) score was assessed. The 4 AIMs categories (limb, axial, orolingual, and locomotive) were scored using a validated rating scale [26, 27] for 1 minute every 20 minutes for 2 hours (total 4 observations; maximal score for each observation, 16; maximal total score per session, 64) performed by a trained investigator as previously described [8, 12, 19, 20, 28-30].

On the 32th day, animals received a 6mg/kg L-dopa injection 1h before a 500nl Daun02 injection (4 µg/µL in 5% DMSO, 5% Tween-80 in PBS at 0.5 µl/min) [11] in the dlBST under light isoflurane anesthesia before being placed in their home cage for 3 days as described [11, 15, 16]. From the 3rd day after Daun 02 injection, all rats received a daily 6mg/kg L-Dopa injection and AIMs were scored [11]. To ensure reversibility of Daun02-induced inactivation, a control solution (5% DMSO, 5% Tween-80 in PBS at 0.5 µl/min) was injected in the same animals 6 days after Daun02 injection and AIMs were evaluated.

At the end of the Daun02 experiment, 1 hour after the last L-DOPA injection, i.e. at the peak of behavioural effect, rats were deeply anesthetized with chloral hydrate (400mg/kg, i.p., VWR) and perfused transcardially with 0.9% NaCl followed by ice-cold 4% formaldehyde in PBS. Brains were removed, postfixed overnight in the same fixative (4°C), then cryoprotected for 48h at 4°C in 20% PBS-sucrose. Brains were frozen in isopentane at -45°C and stored at -80°C until sectioning.

In vivo electrophysiological validation of the Daunorubicin-induced neuronal electrical inhibition in dlBST neurons

Stereotaxic surgery for *in vivo* electrophysiology, stimulation and recording protocols were performed as described above and previously [21]. Local delivery of Daunorubicin (4µM and 8µM) or its vehicle (PBS) was performed using double barrel pipettes as previously described [21]. Each cell was tested with 100nL of Daunorubicin or the vehicle. At the end of each recording experiment, the electrode placement was marked with an iontophoretic deposit of sky blue dye (-20µA, 15min). To mark electrical stimulation sites, +50µA was passed through

the stimulation electrode for 1min30. Brains were frozen in isopentane and cut with cryostat (30µm thick). Slices were mountained with DAPI vectashield medium and observed at epifluorescent microscopy and transmission microscopy.

Cytochemical detection of β-galactosidase

50µm-thick cryostat-cut coronal rat brain sections were collected, washed twice in PBS and incubated overnight at 37°C in freshly prepared staining buffer [1mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 5mM K3Fe[CN]6, 5mM K4Fe[CN]6, and 2mM MgCl2 in PBS, pH 6.0] as previously performed [11]. Brain sections were washed with PBS, counterstained with neutral red and examined at ×10 and x40 magnification.

Monkey experiments

The animal was first rendered parkinsonian with MPTP-hydrochloride (0.2mg/kg, i.v., Sigma) dissolved in saline as previously described [24, 31-33]. Assessment of parkinsonism was performed in home cage for 30 min by two blinded observers using a validated rating scale [24, 31-33] assessing tremor, general level of activity, body posture (flexion of spine), vocalization, freezing and frequency of arm movements and rigidity (for each upper limb). Following stabilization of the MPTP-induced syndrome (3 months), the animal received twice-daily 20 mg/kg of L-Dopa p.o. for 3 months (Modopar; Roche, Welwyn Garden City, UK; L-Dopa/carbidopa ratio, 4:1) and developed severe and reproducible dyskinesia [3, 12, 13, 41-43]. Once the animal was stably dyskinetic, stereotactic delivery of FosB-LacZ lentiviral vector was conducted under isoflurane anesthesia as previously described [11-13] in the dlBST. Horsley-Clarke stereotaxic technique coupled with ventriculography were used [11] to determine the position of left and right dlBST. A total volume of 30 µL of FosB-LacZ lentivirus was injected bilaterally into one monkey (15 µL per hemisphere: AP +1; ML +/- 2; DV +2 from anterior commissure (AC)) with a Hamilton syringe mounted into a microinjector system (Kopf, California) [12, 13]. Guide cannulas (AP +1; ML +/- 2; DV +4 from AC) were cemented to the skull as previously described [34-36].

Monkey's behavior was recorded OFF and ON L-dopa before, while being exposed (3-5 days after intradlBST injection) and after (7 days after intradlBST injection) of Daun02 (5 µl per hemisphere at 2 µl/min, 4 µg/µL dissolved in 5% DMSO, 5% Tween-80 in PBS under light isoflurane anesthesia) [11]. Each time, it was first recorded in the OFF state for 60 min in an observation cage (dimensions - 1.1m x 1.5m x 1.1m). L-dopa was then administered, and the

monkey's behavior was recorded for a further 240 min in the observation cage. The total duration of observation was 300 min including drug administration [11].

The parkinsonian condition (and its reversal) was assessed on a parkinsonian monkey rating scale using videotape recordings of monkeys [34, 37]. A score of 0 corresponds to a normal animal and a score above 6 to a parkinsonian animal [37]. The severity of dyskinesia was rated using the Dyskinesia Disability Scale [34, 35, 38] as previously described [3, 12, 13, 39-41]: 0, dyskinesia absent; 1, mild, fleeting, and rare dyskinetic postures and movements; 2, moderate, more prominent abnormal movements, but not interfering significantly with normal behavior; 3, marked, frequent and, at times, continuous dyskinesia intruding on the normal repertoire of activity; or, 4, severe, virtually continuous dyskinetic activity replacing normal behavior and disabling to the animal. The duration of anti-parkinsonian action (i.e. on-time), was defined as the number of minutes for which bradykinesia was absent (i.e. score equal to zero) [11]. In addition, the duration of on-time associated with dyskinesia of varying severity was defined as follows; "good" quality on-time represents the number of minutes for which bradykinesia was zero whilst dyskinesia was either absent or of mild or moderate severity (0-2) [11].

Histological data analysis

50µm-thick cryostat-cut coronal rat brain sections were collected and processed for tyrosine hydroxylase (MAB318, Milipore), ΔFosB (sc-48, Santa-Cruz) and D1R (D2944, Sigma) as previously described [8, 11, 42].

***Ex vivo* electrophysiological experiments:**

Fifty-five rats with unilateral 6-OHDA lesions and chronically treated with L-DOPA or benserazide as previously described were used for brain slices neurophysiology experiments. One hour after their last L-DOPA or benserazide injections, the rats were deeply anesthetized with isoflurane (5% at 5L/min). Their brains were rapidly extracted and kept in iced-cold physiological solution containing (in mM) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 6 $CaCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and, 12.5 D-glucose equilibrated with 95%O_2/5%CO_2. The brains were cut coronal slicing (250 µm) with a vibrating microtome (Leica VT-1000) in the physiological solution maintained at 2°C throughout the slicing procedure. Slices containing the BST were incubated at 34°C for at least 60 min and transferred to a chamber that was constantly perfused (3 ml/min) with the physiological solution maintained at 34°C. Whole-cell voltage-clamp recordings of electrically-evoked AMPA excitatory postsynaptic currents

(EPSC) or $GABA_A$ inhibitory postsynaptic currents (IPSC) were made using glass microelectrodes (3.5MOhm) filled with a solution containing (in mM) 130 K^+-gluconate, 1 EGTA, 5 HEPES, 2 MgATP, 0.3 GTP, and 1 P-creatine. K^+-gluconate was reduced to 70mM and 80mM KCl was added in the internal solution for $GABA_A$-IPSC recordings [43]. Pharmacologically-isolated post-synaptic AMPA or $GABA_A$ currents were evoked by local fiber stimulation with tungsten bipolar electrodes placed in the dlBST, 100-500 μm dorsal from the recorded neurons. Paired electrical stimuli (10-100μA, 0.1ms duration, 20Hz) were evoked at 0.1Hz while neurons were voltage-clamped at -70mV. After 5 mins of stable baseline, the D1R agonist SKF-81297 (1μM) was bath-applied for 5 mins and its effect on AMPA-EPSC or $GABA_A$ was determined. To measure AMPA to NMDA ratios (A:N), the recording electrodes contained (in mM) 130 Cs^+MeSO3^-, 1 EGTA, 5 HEPES, 2 Mg-ATP, 0.3 GTP, and 1 P-creatine. Neurons were initially voltage-clamped at -70mV until stable recordings and gradually depolarized to +40mV to relieve Mg^{2+} block of NMDA currents. After 10 mins of stable baseline, AMPA currents were isolated by bath applying the NMDAR blocker AP-5 (50μM) for 2-5 mins. NMDA currents were obtained off-line by subtracting AMPA EPSC from the total EPSC. The peak of AMPA and NMDA currents were used to calculate A:N ratios. Recordings were made using a Multiclamp 700B amplifier and a Digidata 1440A (Molecular Devices Scientific). Data were acquired and analyzed with Axograph X running on Apple computers. We measured drug-induced change in post-synaptic currents peak amplitude from baseline in percentage (((Peak $amplitude_{drug}$-Peak $amplitude_{baseline}$)/Peak $amplitude_{baseline}$)*100). Data are reported as Mean ± s.e.m. In graphs where time-courses of drug effects are presented, each data point is the average of 1 min bins (6 evoked PSC) across recorded neurons. We calculated paired-pulse ratios (PPR) by dividing the second (S2) by the first (S1) peak amplitude. All statistical analyses were done with JMP 12.0 (SAS Institute Inc.). Stock solution of AP-5 (100mM) was made in double-distilled water. DNQX (100mM) and SKF-81297 (1mM) were prepared in DMSO (100%) and further dissolved in the physiological solution such that brain slices were exposed to DMSO 0.01%.

Data Analysis

Behavioral Data were analyzed with wilocoxon-signed rank t-test [44]. All data are presented as mean ± SEM with a threshold for statistical significance at $p<0.05$. For *in vivo* electrophysiological experiments, cumulative PSTHs of ov/jxBST activity were generated during electrical stimulation of the INS Cx. Excitatory magnitudes ($_{Rmag}$ values) were normalized for different levels of baseline impulse activity. R_{mag} values for excitation were

calculated according to: Excitation R_{mag}=(counts in excitatory epoch)–(mean counts per baseline bin x number of excitatory bins in excitatory epoch). For a comparison between three groups, values were subjected to a one–way ANOVA followed (if significant) by Bonferroni post hoc tests [21].

Results

Electrical inhibition of dlBST ΔFosB-expressing neurons alleviates LID

To directly assess the causal role of dlBST upon AIM severity, in the rodent and non-human primate analog of dyskinesia, we inhibited the electrical activity of dlBST ΔFosB-expressing neurons using the selective Daun02/β-galactosidase inactivation method. This method consists into the local administration of the prodrug Daun02 converted into Daunorubicin by ß-galactosidase, readily expressed in mammalian cells previously transduced with the E. coli LacZ gene under the control of a cell-specific promoter [11, 14-16]. Then, the newly synthesized Daunorubicin is able to decrease neuronal excitability [17]. We recently showed that the electrical activity of striatal neurons is inhibited following both Daun02/ß-galactosidase inactivation or Daunorubicin injection *in vitro* and *ex vivo* [11] . Here, we demonstrate, *in vivo,* that intra-neuronal injection of Daunorubicin is able to drastically decrease the electrical activity of dlBST neurons following INS Cx stimulation in rats (-60%; ***p<0,05 for all; **Figure 1ABCD**). Thus, we could selectively inactivate β-galactosidase transduced neurons following Daunorubicin synthesis.

Therefore, we injected, *in vivo,* a FosB-LacZ lentivirus expressing the ß-galactosidase only in the dlBST FosB/ΔFosB-expressing neurons [11] of 6-OHDA-lesioned rats chronically treated with L-Dopa [8, 12, 13, 45]. After the establishment of stable AIMs, a single intra-dlBST administration of Daun02 significantly decreased AIMs compared to baseline score (*p<0.05; **Figure 1E**). AIMs reduction lasted 3 days compared with baseline score (22%, 21% and 13% respectively; *p<0.05 for all; **Figure 1E**) in keeping with previous demonstration of Daun02-mediated behavioral span [11, 16]. After a return to baseline AIMs score, a control solution, (vehicle without Daun-02), was injected in the dlBST of the same rats. No significant difference in AIMs score was found between vehicle-treated rats and baseline scores while Daun02-inactivation induced a significant decrease in AIMs score compared to vehicle injection for 2 days (19% and 18% respectively; $p<0.05 for all; **Figure 1E**). However, Daun02-inactivation did not induce significant modifications of the rotational behavior, an index of the anti-parkinsonian effect of L-Dopa, compared with both baseline and control-

treated rats (**Figure 1F**). ß-galactosidase staining confirmed an expression of the FosB-LacZ lentivirus restricted to the dlBST region **(Figure 1G)**.

Our results highlight that Daun02-induced inactivation of extra-striatal FosB/ΔFosB-expressing neuron excitability significantly alleviates AIMs in dyskinetic 6-OHDA-lesioned rats. In order to translate these findings into a more clinically relevant context, we determine whether such approach can revert already established dyskinesia in an animal model that better recapitulates the human condition. As a proof of concept, we thus investigated the behavioral impact of the Daun02 inactivation method in the gold standard experimental model of LID, the MPTP-lesioned L-Dopa–treated macaque monkey [3, 12, 13, 41, 46, 11]. A L-Dopa–treated dyskinetic macaque received the FosB-lacZ lentivirus in the dlBST. Parkinsonian disability scores in both the OFF (before L-dopa administration) and ON states (after L-dopa administration), and LID scores in the ON state were indistinguishable between observations made before and 8 weeks after the delivery of FosB-lacZ lentivirus. When injected in the dlBST, Daun02 decreased the dyskinesia score (**Figure 2A**) without affecting the disability score (**Figure 2B**), resulting in an increased 'good on-time' period (**Figure 2C**). The monkey returned to its pre-surgery dyskinesia (**Figure 2A)** score 4 days later.

Taken together, these results demonstrate that Daun02-induced electrical inactivation of dlBST FosB/ΔFosB-expressing neurons decreases LID severity in rats and, as a proof of concept, in a clinical relevant model of LID: one dyskinetic MPTP-treated macaque.

LID induce changes in D1R modulation of oval BST inhibitory synaptic transmission

In order to identify the neuronal mechanisms involving the dlBST in LID pathophysiology, we analysed the excitatory and inhibitory synaptic transmission both in the ovBST and jxBST of dyskinetic 6-OHDA lesioned rats. Chronic L-Dopa treatment did not change the strength of excitatory synapses in either ovBST or jxBST as measured by AMPA/NMDA ratios (**Figure 3A**). However, we found an increased expression of the dopaminergic D1 receptor (D1R) exclusively in the dlBST of dyskinetic rats, which co-localized with ΔFosB. (**Figure 3B**). We therefore postulated that the dlBST could impact LID severity through a D1R-related mechanism. Consequently, we applied the D1R agonist SKF-81297 on brain slice of dyskinetic and control 6-OHDA-lesioned rats followed by measurement of AMPA and $GABA_A$ current. In the jxBST, no modification of D1-modulated AMPA-mediated EPSC (**Figure 3C)** and GABAa-mediated IPSC (**Figure 3D)** was found between the 2 groups. However, in the ovBST, while there is no difference in AMPA-mediated EPSC (**Figure 3E)**, we found a significant enhancement in D1 modulation of GABAa-mediated IPSC of

dyskinetic 6-OHDA-lesioned rats (**Figure 3F)**. Altogether, these results demonstrate, for the first time, an extra-striatal alteration of the GABAa-mediated inhibitory synaptic transmission in the ovBST induced by a chronic L-Dopa treatment.

Discussion

More than fifty years after its introduction in clinical therapy, L-Dopa remains the gold standard treatment for PD but rapidly induces fluctuations and LID. Those latter have been associated with both presynaptic and postsynaptic striatal mechanisms [24, 25]. In the present study, we unravelled new striking insights of the involvement of an extra-striatal brain region: the dlBST in LID pathophysiology. First, we demonstrated that the electrical inhibition of dlBST FosB/ΔFosB neurons decreases LID severity. Remarkably, as a proof of concept, we confirmed the dlBST involvement in LID pathophysiology in the gold standard model of LID: the dyskinetic MPTP-treated macaque. Then, we unravelled a specific and significant increase in D1 modulation of $GABA_A$-mediated inhibitory synaptic transmission only in the ovBST of dyskinetic rats. Altogether, these results demonstrate, for the first time, a translational validation of the functional involvement of the dlBST in LID, underlying a putative key role of structures outside of the basal ganglia in LID pathophysiology.

The dlBST receives robust monoaminergic inputs featuring serotonin (5-HT), noradrenealine (NA) and dopamine (DA) [47]. The dlBST DA inputs originate from the ventral tegmental area (VTA), the periaqueducal gray region and the retrorubral field. They form a fairly diffuse input to the dlBST with dense DA terminal fields in the ovBST and the jxBST [48-50]. In addition, the dlBST is innervated by the amygdala, the hippocampus and the prefrontal cortex [51]. Interestingly, previous studies demonstrated that the monoaminergic neurochemistry of the amygdala, prefrontal cortex and IEG-expression pattern of hippocampus are altered in PD and LID animal models [4, 8]. These intriguing results suggest that this network, mainly involve in affective, cognitive and motivational disorders, could also impact LID severity directly or indirectly.

LID derive in part from sensitized D1 receptors due to chronic L-Dopa stimulation [12, 52]. Recent studies showed a pathological-related cell-surface expression, sensitivity and trafficking of the striatal D1R in LID pathophysiology both in rodents and non-human primates [19, 52, 53, 12], ascertaining a crucial role of sensitized-D1R in LID. In addition, LID disturb striatal D1R signalling pathway [54-61] inducing, among others, alterations in IEG expression, especially for ΔFosB [58, 62, 63], which impacts LID severity [9-11]. Interestingly, the dlBST shares similar striking events with the striatum, as an increase in D1R expression

induced exclusively by a chronic L-Dopa treatment, which co-localized with ΔFosB. Then, we unravelled a LID-related increase in $GABA_A$-mediated inhibitory synaptic transmission specifically modulated by D1R. Interestingly, recent studies demonstrated that ovBST D1R is involved in DA-related disorder, especially in the field of drug addiction [64, 65], also associated with an increase in D1R-modulated $GABA_A$-mediated inhibitory synaptic transmission [66]. Altogether, our data demonstrate a D1R-related mechanism engaging the dlBST in LID pathophysiology through both D1R/ΔFosB-expressing neurons and D1R-driven inhibitory synaptic transmission.

Conclusion

The present study described the involvement of an extra-striatal structure: the dlBST in LID. Even if the underlying mechanisms involving the dlBST in LID pathophysiology are not yet completely elucidated, our data suggest that these effects should be mediated, at least in part, by D1R/ΔFosb expressing neurons and D1R/$GABA_A$-mediated inhibitory synaptic transmission. Taken altogether, our results highlight for the first time the functional role of the dlBST in LID, both in rat and non-human primate, offering a new target to innovative treatments of LID.

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Figure legends:

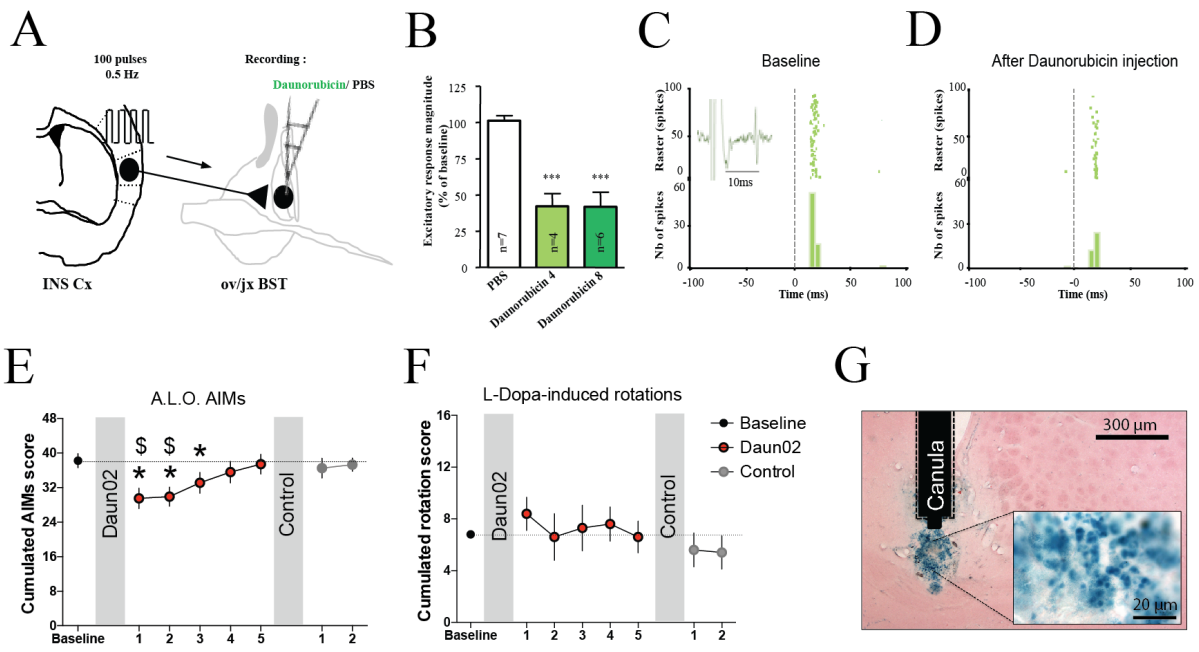

Figure 1: Daun02-induced inactivation of dlBST ΔFosB-expressing neurons alleviates LID in rats. **A**- INS Cx stimulation and ov/jxBST recording protocols. **B**- Quantitative analysis of inhibitions induced by Daunorubicin infusion (Daunorubicin 4: 4µg/µL; Daunorubicin 8: 8µg/µL) on excitatory responses evoked by the INS Cx stimulation. Only neurons responding to Daunorubicin have been included in this analysis (4 out of 7 for Daunorubicin 4 and 6 out of 6 for Daunorubicin 8) (*** $p<0.001$ from PBS). **C**- Typical PSTHs and associated rasters showing responses of ov/jx BNST neurons before daunorubicin infusion. Stimulus at t_0 (gray line). Bin width, 5 ms. Representative electrophysiological trace in inset. **D**- Typical PSTHs and associated rasters showing responses of ov/jx BNST neurons after Daunorubicin (4µg/µL) infusion. Stimulus at t_0 (gray line). Bin width, 5 ms. **E**- Cumulated axial, limb and orofacial (A.L.O.) AIMs scores in L-Dopa-treated 6-OHDA rats (n=10) before and after Daun02 and after control solution injection (* $p<0.05$ from baseline and $ $p<0.05$ from control). **F**- Cumulated rotation scores in L-Dopa-treated 6-OHDA rats (n=10) before and after Daun02 and after control solution injection. **G**- Representative dlBST cytochemical detection of ß-galactosidase expression in the Daun02-injected side of dyskinetic rats (scale bar: 300µm) with an inset (scale bar: 20µm).

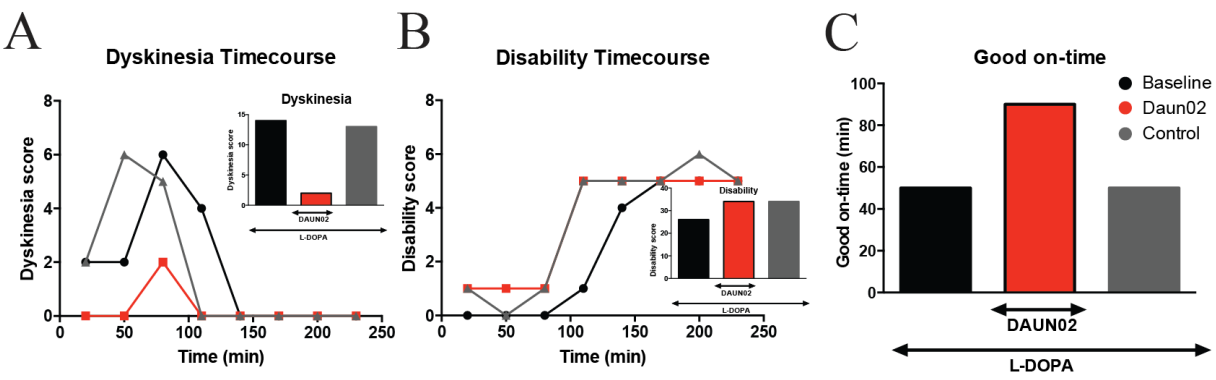

Figure 2: Daun02-induced inactivation of dlBST ΔFosB-expressing neurons reduces LID severity in non-human primates and spares L-Dopa beneficial effect. **A**- Cumulated dyskinesia score in a L-Dopa-treated MPTP-lesioned macaque before and after Daun02 injection. **B**- Cumulated disability score in a L-Dopa-treated MPTP-lesioned macaque before and after Daun02 injection. **C**- Cumulated good on time of a L-Dopa-treated MPTP-lesioned macaque before and after Daun02 injection.

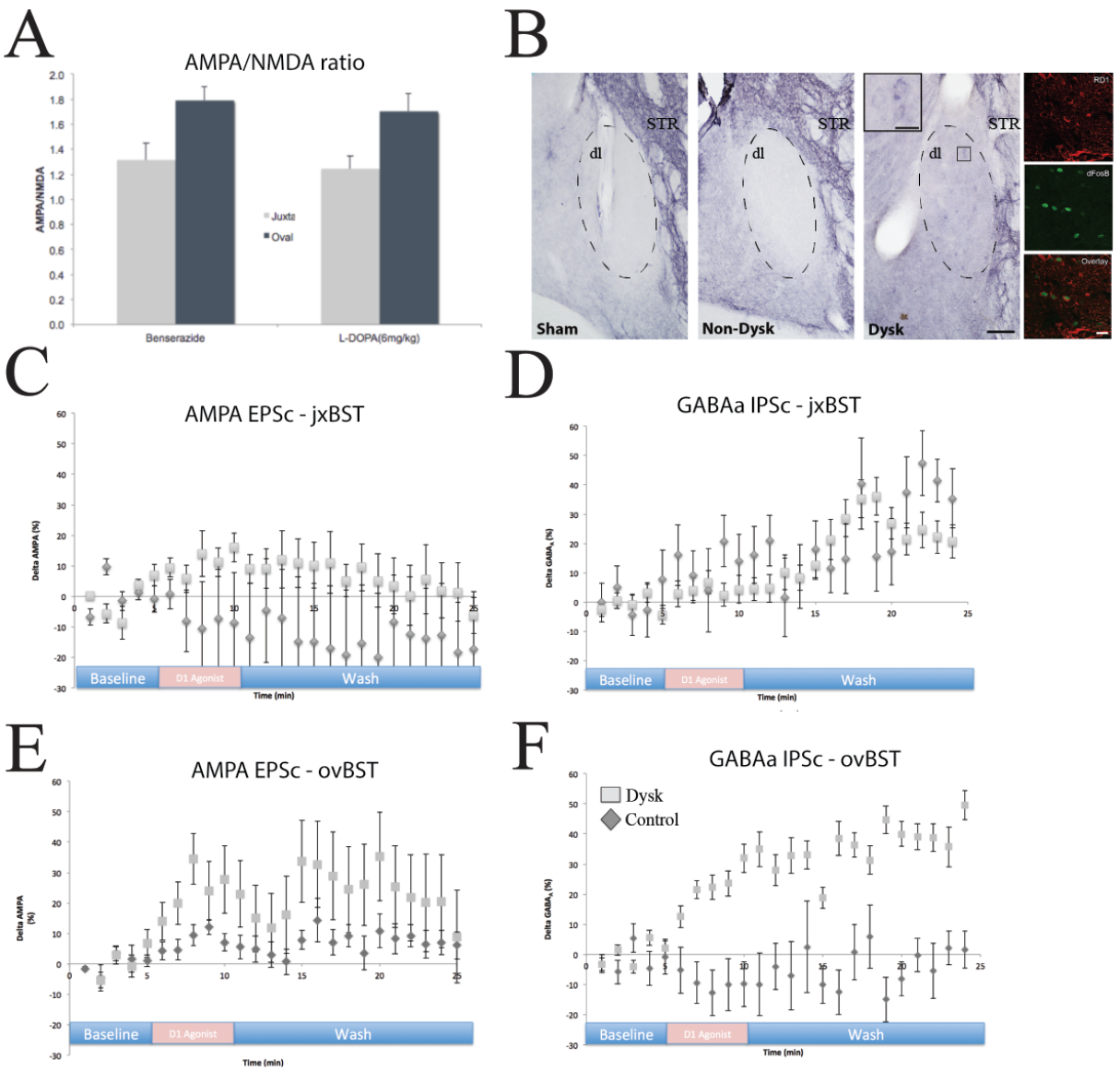

Figure 3: Chronic L-Dopa treatment alters the $GABA_A$ mediated synaptic transmission in the oval nucleus of the dorsolateral BST. **A-** AMPA/NMDA ratio in the ov/jx BST of dyskinetic 6-OHDA lesioned rats (n=30) and control benserazide-treated 6-OHDA lesioned rats (n=25). **B-** Representative dlBST mapping of D1R expression (dashed lines) in sham-operated (sham), 6-OHDA-lesioned (Non-Dysk) and L-Dopa-treated dyskinetic 6-OHDA-lesioned rats (Dysk) (scale bar: 300µm) with representative insets (scale bar: 20µm) showing D1R, ΔFosB and co-localization of D1R/ΔFosB expression (dl = dlBST; STR = Striatum). **C-** Effect of a 5 min bath application of the D1R agonist SKF-81297 on the amplitude of electrically evoked jxBST AMPA-EPSC (0.1 Hz) as a function of time in dyskinetic 6-OHDA-lesioned rats (n=30) (Dysk) and benserazide-treated 6-OHDA lesioned rats (n=25) (control). **D-** Effect of a 5 min bath application of the D1R agonist SKF-81297 on the amplitude of electrically evoked jxBST $GABA_A$-IPSC (0.1 Hz) as a function of time in dyskinetic 6-OHDA-lesioned rats (n=30) (Dysk) and benserazide-treated 6-OHDA lesioned rats (n=25) (control). **E-** Effect of a 5 min bath application of the D1R agonist SKF-81297 on the amplitude of electrically evoked ovBST AMPA-EPSC (0.1 Hz) as a function of time in dyskinetic 6-OHDA-lesioned rats (n =30) (Dysk) and benserazide-treated 6-OHDA lesioned rats (n=25) (control). **F-** Effect of a 5 min bath application of the D1R agonist SKF-81297 on the amplitude of electrically evoked ovBST $GABA_A$-IPSC (0.1 Hz) as a function of time in dyskinetic 6-OHDA-lesioned rats (n=30) (Dysk) and benserazide-treated 6-OHDA lesioned rats (n=25) (control). Evoked events were binned (1 min, 6 events) and data points and error bars represent means +/-SEM across all recorded neurons within each experimental group.

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5. Publication 5: Striatal NELF-mediated RNA polymerase II stalling controls L-Dopa induced dyskinesia

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Submitted

In the previous studies, we demonstrated that inactivating ΔFosB-expressing neurons decrease LID severity both in rodent and macaques, in the striatum or in structures outside of the basal ganglia. These data highlight the key role of IEG-expressing neurons in LID manifestation. However, the intrinsic transcriptional mechanisms inducing a rapid IEG expression and involving IEGs in LID remain unclear. Recent evidences suggest that expression of many IEGs depends on a prior recruitment of the RNA polymerase II, which initiates transcription elongation and stalls after transcribing a short piece of mRNA near the promoter. RNA polymerase II stalling is critically regulated by a protein complex, the negative elongation factor (NELF), composed of four essential subunits: NELF-A, -B, -C/D and –E. NELF-mediated RNA polymerase II stalling on IEG promoters poises them for rapid transcription within few minutes after an external stimulus. In this study we demonstrated that decreasing NELF-E levels, and hence stalling, is able to achieve both antidyskinetic and potentiation of L-Dopa-mediated antiparkinsonian effect associated with a decrease in ΔFosB, ARC and Zif268 expression. Therefore, our results highlight key the role of IEG transcriptional-related mechanisms in LID establishment, acute LID manifestation and in the therapeutic response to L-Dopa.

Striatal NELF-mediated RNA polymerase II stalling controls L-dopa induced dyskinesia

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Abstract

Long-term L-Dopa treatment leads to involuntary aimless movements called L-Dopa-induced dyskinesia (LID). L-Dopa treatment induces an overexpression of several molecular markers in the striatum, in particular the members of the immediate-early gene (IEG) family. Their rapid transcription involves the stalling of RNA polymerase II on IEG promoters, a mechanism that critically depends on the presence of the negative elongation factor (NELF) protein complex.

We therefore hypothesized that reducing stalling could (i) decrease IEG expression and (ii) positively impact the severity of LID. To assess the precise role of NELF-mediating RNA polymerase II stalling in LID, we depleted the key NELF-E subunit using lentiviral vector delivery of a short hairpin RNA (shRNA) in the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats. NELF-E mRNA silencing significantly (i) attenuated the development of abnormal involuntary movements (AIMs) in response to chronic L-dopa treatment and (ii) reduced AIMs severity when established. In both experimental designs, NELF-E mRNA silencing significantly increased the antiparkinsonian response to L-Dopa. Effectiveness of silencing was demonstrated by the significant decrease in striatal ΔFosB, ARC and Zif268 IEG expression.

Repression of NELF-mediating RNA polymerase II stalling thus achieves both antidyskinetic and potentiation of antiparkinsonian L-Dopa effect, highlighting the role of transcriptional events in LID establishment, acute LID manifestation and in the therapeutic response to L-Dopa.

Introduction

The most effective symptomatic therapy in Parkinson's disease (PD) remains the dopamine precursor L-3,4-dihydroxyphenylalanine (L-Dopa). Long-term treatment leads to involuntary aimless movements called L-Dopa-induced dyskinesia (LID) (Stocchi et al., 1997; Fahn, 2008), which first causative event is a L-Dopa-induced striatal overexpression of several molecular markers, in particular the members of the immediate-early gene (IEG) family, a class of genes rapidly transcribed in response to an external stimulus, including ΔFosB, ARC and Zif268 (Gerfen, 1990; Gerfen et al., 1995; Berke et al., 1998; McClung et al., 2004; Bastide et al., 2014). Down-regulating expression of ΔFosB for instance, decreases LID severity both in rodent (Andersson et al., 1999) and non-human primates (Berton et al., 2009). While the mechanisms of rapid IEG transcription remain unclear, recent evidences suggest that expression of many IEGs depends on a prior recruitment of the RNA polymerase II, which initiates transcription elongation and stalls after transcribing a short piece of mRNA near the promoter (Lis, 1998; Nechaev and Adelman, 2011; Saha et al., 2011; Saha and Dudek, 2013). RNA polymerase II stalling is critically regulated by a protein complex, the negative elongation factor (NELF), composed of four essential subunits: NELF-A, -B, -C/D and –E (Narita et al., 2003; Saha et al., 2011; Saha and Dudek, 2013). NELF-mediated RNA polymerase II stalling on IEG promoters poises them for rapid transcription within few minutes after an external stimulus (Saha et al., 2011). While the *in vitro* machinery is well described, the role of NELF-mediated RNA polymerase II stalling remains however to be demonstrated in vivo in physiological and pathological states.

Therefore, to assess the precise role of NELF-mediated RNA polymerase II stalling upon LID severity, we depleted the NELF-E subunit by RNA interference (RNAi), using a lentiviral (LV) vector delivering a short hairpin RNA (shRNA) in the striatum of dyskinetic 6-hydroxydopamine (6-OHDA) lesioned rats and we quantified the L-Dopa induced abnormal involuntary movements (AIMs), the rodent analog of LID. We then quantified the striatal expression of ΔFosB, ARC and Zif268 to assess the impact of NELF-mediated RNA polymerase II stalling on IEG expression.

Material and Methods

Design of LV vectors and NELF-E knockdown validation

shRNA LV plasmids (pLKO.1) (Dehay et al., 2012) carrying pre-designed short hairpins sequences for NELF-E or scrambled hairpin sequences were purchased from Sigma–Aldrich (USA). The shRNA sequence used to target NELF-E mRNA is: CCG GCT GGA TTC CTT GTG CCT CAT ACT CGA GTA TGA GGC ACA AGG AAT CCA GTT TTT G (TRC Number: TRCN0000074958) (Saha et al., 2011). Lentiviral production (final titer of 2.55 x 10^8 pI/ml) was performed in the IFR 66 vectorology platform (University of Bordeaux, France) by transfection with a 3 viral vector system: shRNA LV plasmids (NELF-E or scramble), pCMV-Δ8-9 (encapsidation plasmid), and VSV-G (cDNA encoding the envelope glycoprotein of vesicular stomatitis virus) in FT-HEK293 cells (Porras et al., 2012).

To determine the level of NELF-E knockdown, rat striatal primary cell cultures were infected by both LV shRNA NELF-E (n=3) and scramble (n=3). Rat striatal cell cultures were prepared from E15 rat brains as previously described in details (Martin-Negrier et al., 2006; Berthet et al., 2012; Dehay et al., 2012). Striatal neurons were infected at DIV14 with 2µL of LV shRNA NELF-E or scramble. Cells were washed with cold PBS at 4 °C and lysed in buffer containing 25 mM Tris·HCl (pH 6.8), 1% SDS, 250 mM DTT, 7.5% glycerol, and 0.05% bromophenol blue. For immunoblotting, 40 µg of protein was loaded per lane and separated on 18% SDS/PAGE, transferred to nitrocellulose membranes and immunoblotted with rabbit anti-NELF-E (1:500; Millipore ABE-48) and mouse anti-tubulin (1:1000; Sigma T5168) was used to control equal loading as previously described (Dehay et al., 2010; Dehay et al., 2012). LV shRNA NELF-E induced a 47% decrease of NELF-E protein levels ($p<0.05$) **(Figure 1)**.

Behavioural experiments

Experiments were performed in accordance with the European Union directive of September 22, 2010 (2010/63/EU) on the protection of animals used for scientific purposes. The Institutional Animal Care and Use Committee of Bordeaux (CE50) approved the experiments under the license numbers 5012099-A.

Stereotaxic procedure

Adult Sprague-Dawley male rats (175-200g, Charles River Laboratories, Lyon, France) were housed under standard laboratory conditions in a 12-hour light/dark cycle with free access to food and water. On Day 0, unilateral injection of 6-OHDA (2.5 µl at 3µg/µl) was performed in the right medial forebrain bundle (AP=-3.7mm; ML=+1.6mm; DV=-8mm relative to

Bregma (Paxinos and Watson, 2007)), in rats treated 30 minutes before with citalopram (1mg/kg i.p.) and desipramine hydrochloride (20mg/kg i.p.) as previously described (Berthet et al., 2009; Porras et al., 2012; Bastide et al., 2014). 5 µl of the concentrated LV (LV shRNA NELF-E and scramble) were injected into the right striatum at the following coordinates (AP=+0.2mm; ML=+3.5mm; DV=-5.7mm relative to Bregma (Paxinos and Watson, 2007; Porras et al., 2012)). LV were injected either at the same time of the 6-OHDA lesion (**Design 1** : shRNA NELF-E n=9 ; shRNA scramble n=8) or after 6-OHDA lesion and induction of AIMs with chronic L-Dopa treatment (**Design 2 :** shRNA NELF-E n=12 ; shRNA scramble n=9).

Behavioural assessment

Rats displaying an impaired stepping test (Berthet et al., 2009; Bastide et al., 2014), assessed on day 18 post 6-OHDA lesion, and a loss of tyrosine hydroxylase-immunopositive fibers in the striatum greater than 95% were retained for final analysis. From day 21 onwards after the 6-OHDA lesion, all rats (experimental designs 1 and 2) received once daily an i.p. injection of a combined dose of benserazide (15mg/kg) and L-Dopa (6mg/kg) for 10 days. On day 31, AIMs score was assessed for all animals. While Design 1 animals (adopting a *de novo* protocol) had received LV at time of surgery, Design 2 animals were then stereotaxically injected with LV (LV shRNA NELF-E and scramble) in the striatum as described above on day 34. Design 2 rats were allowed to recover for 9 days before resuming the L-Dopa injection schedule. They were then tested for AIMs for the next 10 days and scored on the last day of testing. The 4 AIMs categories (limb, axial, orolingual, and locomotive) were scored using a validated rating scale (Cenci et al., 1998; Lundblad et al., 2002) for 1 minute every 20 minutes for 2 hours (total 4 observations; maximal score for each observation, 16; maximal total score per session, 64) performed by a trained investigator as previously described (Berthet et al., 2009; Porras et al., 2012; Bastide et al., 2014).

Tissue preparation

1 hour after the last L-Dopa injection, i.e. at the peak of behavioural effect, rats were deeply anesthetized with chloral hydrate (400mg/kg, i.p., VWR) and perfused transcardially with 0.9% NaCl followed by ice-cold 4% formaldehyde. Brains were removed, postfixed overnight in the same fixative (4°C), then cryoprotected for 48h at 4°C in 20% sucrose. Brains were frozen in isopentane at -45°C and stored at -80°C until sectioning.

Immunohistochemistry

50µm-thick cryostat-cut coronal rat brain sections were collected and processed for ΔFosB (sc-48, Santa-Cruz), ARC (sc-15325) and Zif268 (sc-189) immunohistochemistry as previously described (Engeln et al., 2012; Bastide et al., 2014). High-resolution image acquisition was performed using a Hamamatsu NanoZoomer 2.0HT at 20x and images were processed with Mercator Pro software (ExploraNova, v7.9.8) for quantification. The boundaries of the striatum were first delineated at low magnification (x 2.5) and threshold quantification based on the immunostaining signal was performed at high magnification (x 20) at the striatal LV injection point to assess ΔFosB, ARC and Zif268 levels of expression. The same threshold was applied for both shRNA NELF-E (n=4) and scramble (n=4) conditions. An investigator blind towards experimental conditions performed the measurements (Bastide et al., 2014).

Data analysis

Behavioural data (AIMs and rotation scores) were analysed using Kruskal-Wallis followed by Dunn's multiple-comparison test (Porras et al., 2012). Western blot and immunohistochemistry data were analysed with 2-tailed unpaired *t-test* (Porras et al., 2012). All data are presented as mean ± SEM with a threshold for statistical significance at $p<0.05$.

Results

Down-regulating NELF-E reduces development of AIMs

To directly determine the causal role of NELF-mediated RNA polymerase II stalling upon establishment of LID, striatal shRNA injections were performed in 6-OHDA-lesioned rats, the rodent analog of PD, not yet exposed to L-dopa (Design 1). AIMs were then induced by chronic L-dopa treatment (Porras et al., 2012). Both AIM severity and rotations were rated on day 10 **(Figure 1A-B)**. Interestingly, NELF-E depletion induced a significant reduction in the development of AIMs compared to shRNA scramble condition ($p<0.05$) **(Figure 1A)**. In addition, L-Dopa-induced rotations, an index of the L-Dopa anti-parkinsonian effect, were increased by 77% ($p<0.05$) compared to shRNA scramble condition **(Figure 1B)**.

Down-regulating NELF-E reduces severity of established AIMs

We next analysed the behavioural impact of NELF-E depletion upon LID manifestation by injecting LV shRNA NELF-E and scramble in the striatum after the establishment of AIMs in

6-OHDA-lesioned rats (Design 2) (Porras et al., 2012) **(Figure 1C-D)**. No significant modification was found both in AIM severity and number of contraversive rotation scores between the conditions before LVs injection and after LV shRNA scramble injection (not shown). In keeping with our working hypothesis, NELF-E knockdown induced a significant decrease in L-dopa-induced AIM severity compared to the condition before LV shRNA NELF-E injection ($p<0.05$) **(Figure 1C)** on the 10^{th} day of L-Dopa treatment. Moreover, L-Dopa-induced rotations were increased after L-Dopa injection when NELF-E is depleted in comparison to the condition before LV shRNA NELF-E (+70%, $p<0.05$) **(Figure 1D)**.

Taken together, our data indicate that decreasing NELF-E levels, and hence stalling, reduces sensitization process (Design 1) and reduces established AIMs (Design 2) while enabling better therapeutic response to L-Dopa (Designs 1 and 2).

Down-regulating NELF-E reduces IEG expression

ARC, ΔFosB and Zif268 IEGs, which striatal expression is enhanced in LID and correlates with their severity (Bastide et al., 2014), are thought to be under the control of NELF-mediated stalling (Saha et al., 2011; Saha and Dudek, 2013). We thus quantified their expression following NELF-depletion in the dyskinetic L-dopa-treated 6-OHDA-lesioned rats used in Designs 1 and 2 **(Figure 2)**. Interestingly, NELF-E knockdown in those dyskinetic 6-OHDA-lesioned rats induces a significant decrease in ARC (-61%, $p<0.05$; **Figure 2A-C**), ΔFosB (-69%, $p<0.05$; **Figure 2D-F**) and Zif268 (-70%, $p<0.05$; **Figure 2G-I**) expression compared to the scramble condition, suggesting that NELF-E is indeed responsible of stalling for those IEGs in the rat striatum.

Discussion

L-Dopa, the gold standard treatment for PD, rapidly induces fluctuations and LID. Those latter being associated with both presynaptic and postsynaptic striatal mechanisms (Bezard et al., 2001; Jenner, 2008), including an impressive and rapid overexpression of ΔFosB, ARC and Zif268 IEGs (Gerfen, 1990; Gerfen et al., 1995; Berke et al., 1998; Sgambato-Faure et al., 2005; Bastide et al., 2014). However, the actual transcriptional mechanisms responsible for such IEG enhanced expression remained unclear. In the present study, we focused on the NELF protein complex, which stalls functional RNA polymerase II, inducing an enrichment of the latter upon IEG promoters (Saha and Dudek, 2013). Accordingly, NELF-E downregulation led to significant *in vivo* down-regulation of ΔFosB, ARC and Zif268 expression (**Figure 2**). Interestingly, we showed that the depletion in the NELF essential

subunit NELF-E induced a decrease both in the priming for LID (**Figure 1A-B**) and in the manifestation severity of established LID (**Figure 1C-D**). Such effects were associated with an increase in L-Dopa induced rotations suggestive of a greater therapeutic benefit in the response to L-Dopa (**Figure 1B and D**).

The impact of NELF-mediated RNA polymerase II stalling upon gene expression would be dependent of the genomic background and of the nature of the stimulus that triggers transcriptional enhancement. NELF was originally thought to be a major repressor of transcription events by blocking RNA polymerase II elongation (Yamaguchi et al., 1999; Yamaguchi et al., 2002). *In vitro* NELF transient depletion was found to induced an increase in gene expression following an external stimulus (Aida et al., 2006). However, stable NELF knockdown in different cell lines triggered both an up-regulation and a down-regulation of dozens of genes including IEGs and non-IEGs, uncovering a new positive role of NELF-mediating RNA polymerase II stalling on gene expression (Aiyar et al., 2007; Gilchrist et al., 2008; Fujita et al., 2009; Saha et al., 2011). Interestingly, NELF depletion induced different IEG expression profiles depending on the nature of the stimulus applied (Fujita et al., 2009), suggesting that NELF-mediated RNA polymerase II stalling impacts IEG expression on a specific stimulus-dependant manner.

How NELF-mediated RNA polymerase II stalling controls LID severity remains however enigmatic. A recent study demonstrated that NELF stable knockdown decreases the early transcription of ARC pre-mRNA five minutes after inducing increase in neuronal activity (Saha et al., 2011). However, forty-five minutes after the stimulus, ARC mRNA levels were back to near control condition, reflecting that depletion on polymerase II stalling affect only the onset of transcriptional events (Saha and Dudek, 2013). Interestingly, the peak of LID severity is between 60 and 90 minutes after L-Dopa administration in rodents, non-human primate and patients (Bezard et al., 2001; Cenci et al., 2002; Jenner, 2008; Contin and Martinelli, 2010; Huot et al., 2012). Consequently, an alteration of early transcriptional events, especially for IEGs, could impact or delay the onset of both AIMs and L-Dopa induced rotations which will maintain a down regulation in gene expression, explaining the effect of NELF-E depletion upon LID severity between 30 to 90 minutes after L-Dopa injections associated with a better therapeutic response and a decrease in IEG expression at the peak of LID severity.

The IEG impact upon LID severity has mostly been studied through the modulation of ΔFosB levels. Andersson and co-workers first demonstrated that a down-regulation of ΔFosB by

infusing a *fosb/*Δ*fosb* antisense in the striatum of dyskinetic 6-OHDA-lesioned rats significantly decreased LID severity (Andersson et al., 1999). Remarkably, ΔFosB involvement in LID was confirmed in gold standard model of LID, the dyskinetic MPTP-lesioned macaque, with a striatal viral-mediated overexpression of the ΔFosB dominant negative ΔJunD, which reduces LID without affecting L-Dopa efficacy (Berton et al., 2009). More recently, we demonstrated that the specific inhibition of the electrical activity of striatal ΔFosB expressing neurons both in dyskinetic rat and macaque significantly alleviates LID and increases the L-Dopa benefit effect at the peak of LID severity (Engeln et al., Submitted). LID severity can thus be attenuated through either striatal down-regulation of IEG expression (the present results, Andersson et al., 1999; Berton et al., 2009) or direct inhibition of electrical activity of IEG-expressing neurons (Engeln et al., Submitted). These mechanisms are obviously of different nature although certainly related at least in part. On one hand a direct interference with IEG expression allows acute reduction in AIM severity and one the other hand reduced neuronal excitability of the IEG-expressing neurons enables as well to diminish AIM manifestation. Understanding the relationship between the complex pattern of IEG expression in response to dopamimetic stimulation (Gerfen et al., 1995; Berke et al., 1998) and the resulting medium spiny neuron electrical activity (Engeln et al., Submitted) should be the goal of further experiments.

Conclusion

NELF-mediated RNA polymerase II stalling plays a key role in LID pathophysiology. Our results indicate that decreasing NELF-E levels, and hence stalling, is able to achieve both antidyskinetic and potentiation of L-Dopa-mediated antiparkinsonian effect, highlighting the role of transcriptional events in LID establishment, acute LID manifestation and in the therapeutic response to L-Dopa.

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Figure legends

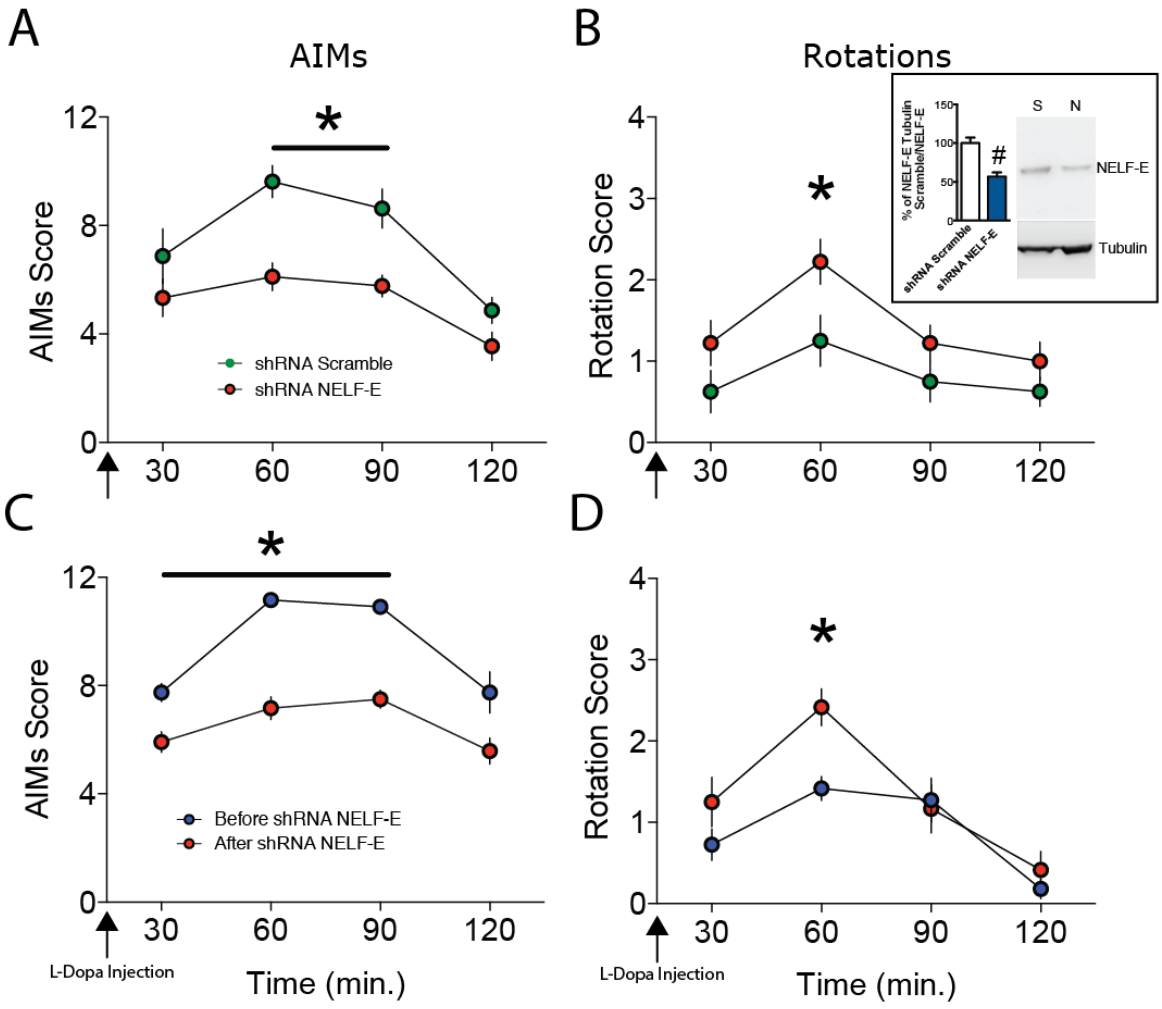

Figure 1. shRNA NELF-E partially prevents AIM development and decreases established AIMs. A-B Experimental design 1, in which animals received either LV shRNA NELF-E (n=9) or LV scramble (n=8) at the same time than 6-OHDA. L-dopa treatment started after down-regulation occurred. Cumulated axial, limb and orofacial AIMs score (**A**) and L-Dopa induced rotation score (**B**) rated from 0 to 120 min after L-dopa administration (Arrow) on day 10 of chronic L-dopa treatment. **C-D** Experimental design 2, in which animals were first lesioned and made dyskinetic before receiving LV shRNA NELF-E (n=12). Cumulated axial, limb and orofacial AIMs score (**C**) and L-Dopa induced rotation score (**D**) rated from 0 to 120 min after L-dopa administration (Arrow) on day 10 of chronic L-dopa treatment before and after LV shRNA NELF-E. *: $p<0.05$. The **inset** in B shows the effects of NELF-E knock down in rat primary striatal neurons upon NELF-E and tubulin protein levels (S = shRNA LV scramble (n=3), N = shRNA LV NELF-E (n=3)). #: $p<0.05$.

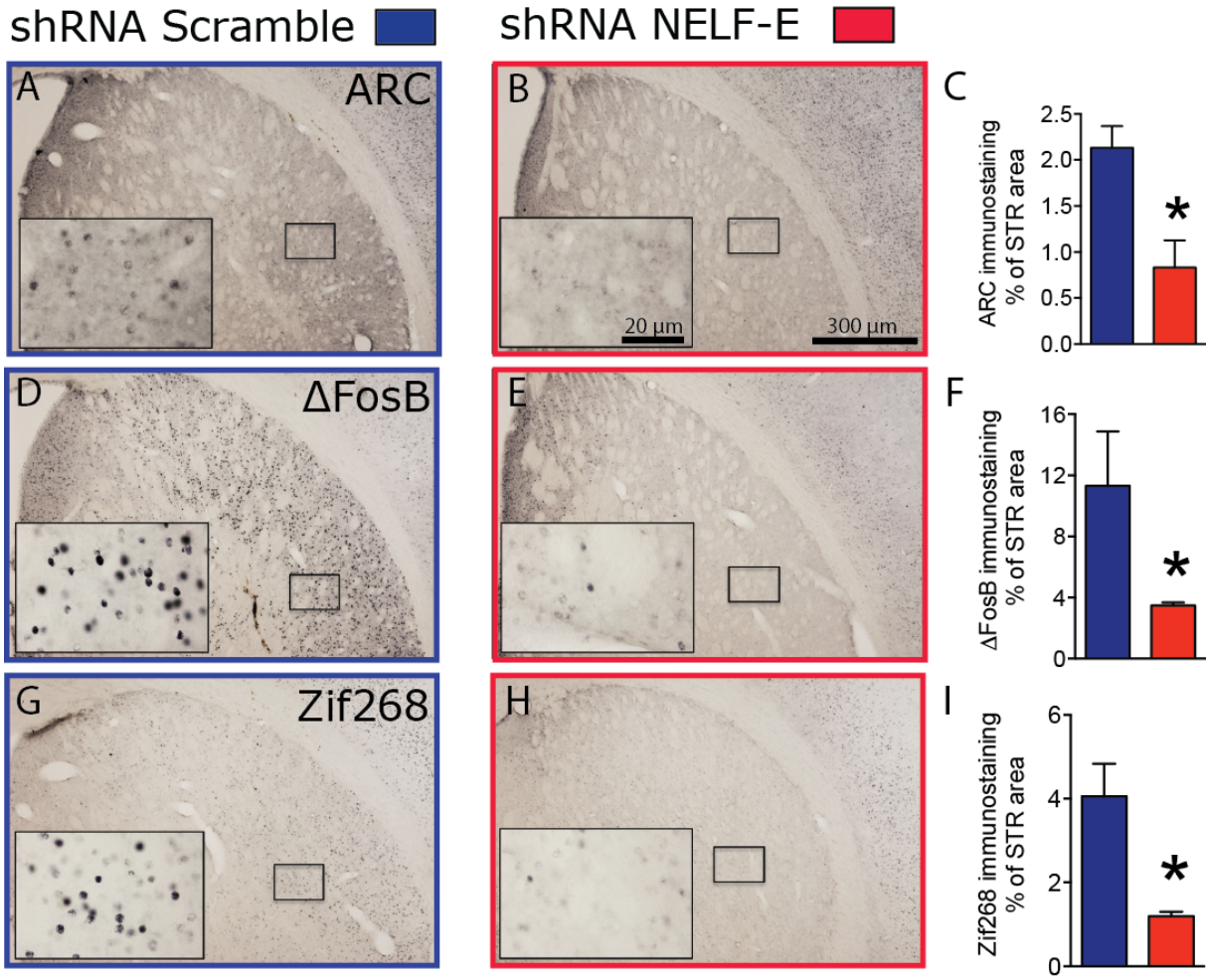

Figure 2. shRNA NELF-E decreases IEG immunostaining in the striatum. Representative examples of IEG striatal (STR) immunostaining in LV shRNA scramble (n=4) (**blue**) and LV shRNA NELF-E (n=4) (**red**) L-dopa-treated 6-OHDA-lesioned rats (scale bar 300µm - with an inset magnification, scale bar 20µm) are shown on the left side while relative threshold quantification results are displayed on the right side (shown as mean ± SEM; *p<0.05). **A-C** ARC immunostaining. **D-F** ΔFosB immunostaining. **G-I** Zif268 immunostaining.

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Discussion

All the results obtained during my PhD are part of a translational research program conducted for many years in the laboratory, starting from experiments in cellular models followed by animals models, including rodents and non-human primates. The objective of this approach is to widen our knowledge in LID pathophysiology to further develop therapeutic strategies.

1. General result statement

During my PhD, we aimed at extending the knowledge of LID pathophysiology by investigating structures potentially affected by a chronic L-Dopa treatment in dyskinetic rodents and non-human primates. The originality of this work resides in a whole brain approach without preconceived notions on the putatively involved structures. First, we demonstrated that the expression of 4 independent IEGs: ΔFosB, ARC, FRA2 and Zif268 is not only significantly modified in the basal ganglia but also in the whole brain of dyskinetic rats compared to non-dyskinetic ones. Such whole brain approach shed light upon 9 structures located outside the basal ganglia displaying a significant overexpression of at least 3 of the studied IEGs. Among the identified nuclei the dlBST (i.e. composed of ovBST and jxBST nuclei), LHb, Pn and CnF display a significant correlation between at least one IEG expression profile and LID severity. Our investigations led, for the first time, to unravel that several structures outside of the basal ganglia are affected by a chronic L-Dopa treatment.

Then, we demonstrated that both dlBST and LHb displayed a LID-related pathological activity at different functional levels including metabolic, electrophysiological and ΔFosB-related transcriptional readouts. Therefore, we confirmed that modified dlBST and LHb neuronal activity in response to L-Dopa is related to LID manifestation. Then, in order to assess if dlBST and LHb neuronal activity might affect LID severity, we inhibited the electrical activity of dlBST and LHb ΔFosB-expressing neurons with a selective inactivation method that we previously validated in the striatum. Interestingly, the inactivation of dlBST and LHb ΔFosB-expressing neurons alleviated LID severity and increased the beneficial effect of L-Dopa in dyskinetic rats. Remarkably, dlBST involvement in LID was confirmed in the gold-standard model of LID, the dyskinetic MPTP-lesioned macaque.

Altogether, the results obtained during my PhD demonstrate, for the first time, the functional involvement of 2 structures outside the basal ganglia in LID.

2. L-Dopa-induced dyskinesia: a side effect involving only the basal ganglia?

The term IEG originated from virology. Following the viral infection of a host cell, several viral genes are rapidly transcribed (Okuno, 2011). This process requires only pre-existing transcription factors already present in the host cell (Watson and Clements, 1980). Based on data collected on cellular differentiation and proliferation in the 80's and 90's, it has become evident that various stimuli, such as growth/differentiation factors, hormones, cytokines or neurotransmitters, induce rapid and transient mRNA synthesis (Almendral *et al.*, 1988; Gerfen *et al.*, 1990; Gerfen *et al.*, 1995; Greenberg and Ziff, 1984; Kruijer *et al.*, 1985). By analogy to the viral IEGs, these genes, which are responsive to extracellular stimuli, are called "cellular" IEGs. The cellular IEGs, simply referred as IEGs, encode many functionally distinct proteins, including structural proteins, signalling molecules, and transcription factors.

In the first study, we mapped the expression of 4 independent IEGs: ΔFosB, ARC, FRA2 and Zif268. The choice of these IEGs was based on pre-existing data. Indeed, ΔFosB, ARC, Zif268 and FRA2 IEGs (Westin *et al.*, 2007; Wirtshafter, 2007) showed a concomitant increased expression in the DA-depleted striatum of rats treated with dopamimetic compounds (Cenci *et al.*, 1999; Ebihara *et al.*, 2011; Sgambato-Faure *et al.*, 2005; Wirtshafter, 2007). Although one would be tempted to make a direct correlation between changes in IEG expression and electrophysiological activity of a considered brain structure, we should bear in mind that this often assumed relationship has not been demonstrated for most IEGs (Loebrich and Nedivi, 2009), especially for those we chose to study. Therefore, increased expression of an IEG should be seen as an increased transcriptional activity and not taken for an increase in electrophysiological activity that remains to be demonstrated. In addition, in this study, the L-DOPA dose was carefully adjusted just below the EC50 value of the L-Dopa (i.e. 3.2mg/kg) for inducing dyskinesia in the majority of animals while still allowing some to not develop any dyskinesia (Putterman *et al.*, 2007). We generated 2 populations of L-Dopa treated rats: dyskinetic 6-OHDA-lesioned rats and non-dyskinetic 6-OHDA-lesioned rats as previously performed in the laboratory (Berthet *et al.*, 2009). Therefore, screening of IEG expression in the whole brain of dyskinetic compared to non-

dyskinetic rats allowed identifying brain nuclei displaying a transcriptional response specifically related to LID. First, this approach allowed us to confirm the overexpression of ΔFosB, ARC, Zif268 and FRA2 in structures classically studied in LID pathophysiology such as: the motor cortex M1, the SNr and the striatum. However, the STN and GPe displayed no IEG immuno-staining both in dyskinetic and non-dyskinetic rats. Then, we identified 9 structures located outside of the basal ganglia displaying an overexpression of at least 3 IEGs in dyskinetic rats including: the dlBST (i.e. composed of ovBST and jxBST nuclei), mBST, rZI, LHb, hippocampus, Pn, CnF and PTg.

In order to strengthen the link between IEG expression and LID, we correlated the number of IEG immuno-postive cells with LID severity for each identified brain nuclei. First, we confirmed the data obtained by Andersson and co-workers (Andersson *et al.*, 1999) by demonstrating a significant correlation between ΔFosB immuno-postive cells and LID severity in the striatum while our study is the first using unbiased stereological methods. Then, we also showed significant correlations in structures outside of the basal ganglia. First, the 2 nuclei of the dlBST showed significant correlations between the intensity of LID and, respectively, the number of ΔFosB immuno-positive cells for the ovBST and FRA2 immuno-positive cells for the jxBST. In the epithalamus, the LHb showed a significant correlation between LID intensity and the number of ARC and ΔFosB immuno-positive cells. Finally, in the brainstem, the Pn and CnF, displayed significant correlations between LID intensity and, respectively, the number of Zif268 immuno-positive cells and FRA2 immuno-positive cells. Therefore, this first study demonstrated that both motor and non-motor domains of cortico-sub-cortical loops showed significant correlations between the number of ΔFosB, ARC, FRA2 and Zif268 immuno-positive cells and LID severity. We are well aware that a correlation does not necessarily imply a causal relationship but might reflect the concomitance of unrelated events.

As previously discussed, IEG expression should be seen as a genomic response following an external stimulus (Okuno, 2011; Perez-Cadahia *et al.*, 2011; Veyrac *et al.*, 2014). Indeed, after the stimulation of a cell-surface receptor, phosphorylated-signalling proteins trigger the activation of transcription factors. Those latter are able to bind IEG promoters and enhance their expression within few minutes after the external stimulation. In addition, recent studies hypothesized that IEG induction can be achieved either by regulating transcription initiation

or by controlling transcription at the level of elongation, as discussed in publication 5 (Saha and Dudek, 2013; Saha *et al.*, 2011). Then, depending on their own functions, IEGs trigger either the expression of effector genes or cellular mechanisms. Therefore IEGs can be seen as a "first wave" of the genomic expression responsible to implement both a global genomic response and cellular modifications inducing changes in neuronal properties accountable for synaptic plasticity.

To confirm the relevance of the IEG screening findings, it should have been necessary to perform the same experiment in the gold-standard model of LID: the dyskinetic MPTP-intoxicated macaque. As the macaque is notably used for final proof of concept of anti-dyskinetic drugs before clinical trials (Iderberg *et al.*, 2012; Morin *et al.*, 2014), this experiment would have reinforced the data obtained in rats, thus supporting the involvement of structures outside of the basal ganglia in LID manifestation.

Then, we further investigated the properties of the previously identified nuclei on LID manifestation. Interestingly, we demonstrate an increase in D1R-mediated long-term potentiation of $GABA_A$-IPSCs in the ovBST of dyskinetic rats (publication 4). Guigoni and co-workers showed that sensorimotor, limbic and associative domains of the basal ganglia and beyond, notably in the BST, display a modified accumulation of 2-DG induced only by a chronic L-Dopa treatment (Guigoni *et al.*, 2005c). Taken together, these data reinforce the potential relationship between a pathological-related activity of the BST and LID manifestation. Then, we demonstrate that LHb displays a decrease in 2-DG accumulation in dyskinetic macaques compared to non-dyskinetic, parkinsonian and sham-operated ones (publication 3). In addition, we show that LHb neuronal firing frequency and pattern are significantly modified by a chronic L-Dopa treatment in dyskinetic rats. Altogether, those data confirm that modified BST and LHb neuronal activity in response to L-Dopa is related to LID manifestation. We will then focus on those 2 nuclei.

3. Demonstration of the causal relationship between the electrical activity of IEG-expressing neurons and LID

The functional role of IEGs, highlighted by a rapid and impressive increased expression following an external stimulus, is far from being well understood, especially in a pathological context. Pathological-related IEG expression seems to occur differentially in distinct neuronal population of a given structure. For instance, repeated administration of L-Dopa to 6-OHDA-lesioned rats normalizes the levels of Zif268 mRNA in striatopallidal neurons, but not in striatonigral ones (Carta *et al.*, 2005). How IEG-expressing neurons are involved in LID pathophysiology remains therefore a mystery.
In this part, we will describe how we succeeded to link the electrical activity of IEG-expression neurons and LID.

Unravelling the precise role of extra-striatal structures in LID pathophysiology required a selective modulation of their electrophysiological activity and the assessment of the impact of such a modulation upon LID severity. In order to assess the causal role of the previously identified nuclei in LID, we used the selective Daun02/ß-galactosidase inactivation method. The Daun02 inactivation method has been originally designed for the treatment of human malignancies (Ajit K. Ghosh, 2000). This method consists into the local administration of the prodrug Daun02 converted into daunorubicin by the ß-galactosidase enzyme, readily expressed in mammalian cells previously transduced with the E. coli *LacZ* gene under the control of a cell-specific promoter (Bossert *et al.*, 2011; Fanous *et al.*, 2012; Koya *et al.*, 2009). Daunorubicin has been shown to reduce calcium ion (Ca^{2+})-dependent action potentials in neuroblastoma cells (Santone *et al.*, 1986). The Daun02 inactivation method was adapted in neuroscience by the group of Bruce Hope in the field of drug addiction and was used in the prefrontal cortex (Bossert *et al.*, 2011; Fanous *et al.*, 2012) and the nucleus accumbens (Koya *et al.*, 2009). Surprisingly, none of these studies validated the daunorubicin-induced inhibition of the neuronal excitability following Daun02 injections. Hence, before using this technique in the 2 nuclei identified with the IEG screening, we had to validate it using: (i) electrophysiology and (ii) behavioural analysis, in a well-known structure involved in LID (publication 2). The striatum appeared to be the perfect choice, as this structure is central in LID pathophysiology (Bezard *et al.*, 2001b; Jenner, 2008).

We first demonstrated that either the application of Daun02 on rat striatal primary neuronal cultures constitutively expressing the ß-galactosidase or the application of Daunorubicin itself on rat striatal brain slices decrease the excitability of medium spiny neurons without affecting their viability. Therefore, this experiment validated the Daun02-induced inhibition of the electrical activity of striatal neurons, giving us the opportunity to use this method *in vivo*.

Among the molecular alterations associated to LID manifestation, an accumulation of ΔFosB was found in the striatum of dyskinetic rodents, non-human primates and human (Berton *et al.*, 2009; Cenci *et al.*, 1999; Tekumalla *et al.*, 2001). Further experiments indicated that ΔFosB is widely involved in the manifestation of long-term behaviour associated to the stimulation of the DAergic system (McClung *et al.*, 2004). Thus, the down-regulation of ΔFosB expression either by molecular interference in rodents or by the overexpression of ΔJunD (i.e. a ΔFosB dominant negative) in non-human primates was able to decrease both the onset (Andersson *et al.*, 1999) and the expression of LID (Berton *et al.*, 2009). Therefore, ΔFosB is not only a marker of LID but also has a functional impact. This led us to express the ß-galactosidase under the control of a FosB promoter in a lentiviral vector to selectively inactivate FosB/ΔFosB-expressing neurons following local Daun02 injection. In accordance with the aforementioned data, we demonstrated that Daun02-induced inhibition of the electrical activity of striatal FosB/ΔFosB-expressing neurons, both in dyskinetic rats and macaques, is able to decrease LID severity without affecting the beneficial effect of L-Dopa. Therefore, our work demonstrates, for the first time, the casual link between the electrical activity of striatal FosB/ΔFosB-expressing neurons and LID severity.

As we demonstrated that the Daun02 inactivation method is able to decrease LID, we therefore used this technique to behaviourally assess the causal role of the dlBST nucleus and LHb in LID manifestation by inactivating FosB/ΔFosB-expressing neurons in these nuclei. We established that the inactivation of the dlBST or LHb of dyskinetic rats decreases LID severity while the anti-parkinsonian effect of L-Dopa was increased only after an inactivation in the LHb. Remarkably, we confirmed the involvement of the dlBST in dyskinetic macaques by a decrease in LID severity without affecting the beneficial effect of L-Dopa after Daun02 injection. Altogether, our results highlight, for the first time, the functional involvement of 2 structures outside the basal ganglia in LID pathophysiology. However, to confirm the relevance of LHb involvement in LID, it will be interesting to inactivate habenular FosB/ΔFosB expressing neurons in dyskinetic non-human primates.

Then, we wanted to know if dlBST or LHb and the striatum are part of a same network involved in LID expression. To do so, we started by inactivating FosB/ΔFosB-expressing neurons both in the striatum and in the dlBST of dyskinetic rats. No additive effect was found and this experiment resulted in a predominant striatal effect, reinforcing the motor component of the striatum in LID. In a second experiment, we should inactivate the striatum and LHb. Then, the double inactivation of dlBST and LHb FosB/ΔFosB-expressing neurons could be more informative of the concomitant impact of 2 extra-striatal structures upon LID pathophysiology.

Next, 2 others extra-striatal structures in the brainstem should be tested to assess their role in LID pathophysiology. Indeed, as previously discussed, Pn and CnF displayed significant correlations between respectively Zif268 and FRA2 immuno-positive cells and LID severity. Therefore, inhibiting the electrical activity of Zif268 and FRA2-expressing neurons respectively in those structures with the Daun02-inactivation method could reinforce our hypothesis of the involvement of structures outside of the basal ganglia in LID pathophysiology.

4. How structures outside of the basal ganglia could impact LID severity?

As previously discussed in publications 1, 3& 4, both dlBST and LHb receive monoaminergic innervation. While the dlBST receives dense DAergic inputs from the VTA, the periaqueducal gray region and the retrorubral field (Freedman and Cassell, 1994; Hasue and Shammah-Lagnado, 2002; Meloni *et al.*, 2006), LHb is mainly innervated by the output structures of the basal ganglia (Haber and Knutson, 2010; Hong and Hikosaka, 2008, 2013) but also receives DA inputs from the VTA (Good *et al.*, 2013; Hnasko *et al.*, 2012; Stamatakis *et al.*, 2013). Indeed, LHb receives excitatory afferents from the border cells of the GPi (Hong and Hikosaka, 2008, 2013), which display an increase firing rate and modified pattern induced by LID (Bezard *et al.*, 2001a). Interestingly, we demonstrated that habenular neurons firing rate, driven by border cells input, is specifically increased in dyskinetic rats (publication 3).

The dlBST projects mainly to the VTA, the paraventricular nucleus of the hypothalamus (PVN) and the lateral hypothalamus (LH) (Dong *et al.*, 2001; Stamatakis *et al.*, 2014).

Therefore, the dlBST is connected to motivational, cognitive and limbic circuits. A previous study performed in the laboratory demonstrated that the prefrontal cortex, the hippocampus and the amygdala displayed a modified monoaminergic neurochemistry in MPTP-lesioned macaques treated with a chronic L-Dopa treatment, supporting the hypothesis of an involvement of cognitive and limbic circuits in LID expression (Engeln *et al.*, 2014). Interestingly, the dlBST receives afferents from those latter (Stamatakis *et al.*, 2014). In our first study we showed a significantly increased expression of the 4 IEGs in the hippocampus. However, we found no modification of the 4 IEG expression pattern in the amygdala of dyskinetic rats in accordance with previous IEG studies (Ebihara *et al.*, 2011). Therefore, studying the monoaminergic neurochemistry in the dlBST of dyskinetic rats and macaques could strengthen and give new insets on the potential involvement of dlBST in LID pathophysiology. LHb can be seen as a crossroad between the limbic system and the basal ganglia (Hikosaka *et al.*, 2008). LHb projects to DAergic areas: VTA and SNc, serotonergic nuclei (i.e. dorsal and medial raphe) and also to the cholinergic laterodorsal tegmentum (Bernard and Veh, 2012; Geisler and Trimble, 2008; Hikosaka *et al.*, 2008; Klemm, 2004). Recent evidence from the field of drug addiction suggests that both dlBST and LHb are involved in DA-related disorders. Interestingly, cocaine administration alters dlBST and LHb neuronal firing and pattern following D1R and D2R stimulation (Krawczyk *et al.*, 2013; Krawczyk *et al.*, 2011; Zuo *et al.*, 2013).
Those data are in accordance with our results in publications 3 and 4 demonstrating a specific L-Dopa-induced modification of the neuronal activity both in dlBST and LHb while inhibition of dlBST or LHb neurons decreases LID severity and increases the beneficial effect of L-Dopa therapy.

Analysing the cellular mechanisms underlying the impact of dlBST and LHb on LID will remain the next step.

Our laboratory confirmed a pathological-related cell-surface expression, sensitivity and trafficking of the striatal D1R in LID pathophysiology both in rodents and non-human primates (Aubert *et al.*, 2005; Berthet *et al.*, 2009; Guigoni *et al.*, 2007). In addition, they demonstrate that the restoration of the D1R trafficking decrease LID severity (Ahmed *et al.*, 2010; Porras *et al.*, 2012a), highlighting the key role of the striatal sensitized-D1R in LID pathophysiology. Interestingly, we showed a concomitant D1R increased expression both in the dlBST and LHb only on the 6-OHDA-lesioned side of dyskinetic rats, unravelling a

potential D1R-related mechanism involved in LID pathophysiology outside of the basal ganglia. In addition, we demonstrated that administration of a D1R agonist (SKF-81297) increases $GABA_A$-IPSCs only in the ovBST nucleus of the dlBST in dyskinetic rats. Ascertaining a role for extra-striatal, e.g. intra LHb or intra BST, D1R may seem provocative. It however shares enough similarity with striatal involvement for being a realistic hypothesis (Bezard *et al.*, 2001b; Cenci *et al.*, 2002; Jenner, 2008). Indeed, as in the striatum, L-Dopa induces an overexpression of ΔFosB in dlBST and LHb, which co-localizes with D1R only on the 6-OHDA-lesioned side of dyskinetic rats. Interestingly, ΔFosB seem to be directly related to the D1R pathway as its expression is directly modified by specific D1R agonist/antagonist (Doucet *et al.*, 1996; Feyder *et al.*, 2011; Westin *et al.*, 2007). Altogether, these data suggest an involvement of extra-striatal D1R/ΔFosB neurons of the dlBST and LHb in LID pathophysiology. To confirm this hypothesis, we plan to assess the neuronal firing frequency and pattern of dlBST and LHb neurons following an application of a D1R antagonist (SCH23390) in dyskinetic rats compared to dyskinetic, 6-OHDA-lesioned and sham-operated ones untreated with SCH23390. Then, we will quantify ΔFosB expression to validate the casual link between D1R stimulation and ΔFosB expression in the dlBST and LHb.

As described in the introductory review, the striatal D1R signalling pathway has been thoroughly studied in LID. Previous studies highlighted LID-related alterations on specific signalling proteins such as increased levels of adenylyl cyclase 5 (Rangel-Barajas *et al.*, 2011) resulting in augmented synthesis of cAMP and hyper-activation of PKA/DARPP-32 associated to an activation of ERK/MSK1, which controls transcriptional and translational processes (Bateup *et al.*, 2010; Feyder *et al.*, 2011; Fieblinger *et al.*, 2014; Lebel *et al.*, 2010; Picconi *et al.*, 2003; Santini *et al.*, 2012; Santini *et al.*, 2010a; Santini *et al.*, 2007). Therefore, to strengthen the hypothesis of an extra-striatal D1R-related mechanism involvement in LID, it will be relevant to investigate the levels and activation status both of trafficking (i.e. GRK6 and PSD-95) and signalling (i.e. PKA/DARPP-32 and ERK/MSK1) proteins of dlBST and LHb D1R neurons in dyskinetic animals models.

How extra-striatal neurons impact LID behaviour remains an open question. The BST is well known to be involved in stress and anxiety (Herman and Cullinan, 1997; Stamatakis *et al.*, 2014; Walker *et al.*, 2003). Interestingly, LID are often described as being triggered or enhanced in patients with PD by emotional factors such as stress, talking in public, or when eating (Voon *et al.*, 2009). Thus, we could make the hypothesis that LID, rather than a simple

medication-related motor manifestation, might also involve a cognitive and limbic pathophysiological basis (Engeln *et al.*, 2014) in which the dlBST could be part of, and impact indirectly LID motor behaviour. As previously discussed, LHb acts as a junction connecting the limbic system and the basal ganglia to the monoaminergic centres. Interestingly, exogenous L-Dopa is mostly uptaken by serotonergic terminals allowing the dopamine to become the "false" neurotransmitter of the serotonin neurons (Carta and Bezard, 2011; Navailles *et al.*, 2010b; Ng *et al.*, 1970b). As the "false" neurotransmitter hypothesis involving the serotoninergic system in LID addresses the presynaptic component of LID pathophysiology (Carta and Bezard, 2011; Navailles *et al.*, 2010b), the efferent connectivity of the LHb suggests that it may play a role in controlling serotonergic output (Bernard and Veh, 2012). Impaired LHb input would thus participate to the aberrant dopamine release from 5-HT terminals and could impact LID severity (Carta *et al.*, 2007; Carta *et al.*, 2008a, b; Navailles *et al.*, 2011a; Rylander *et al.*, 2010b).

5. Potential therapeutics

Considering the Daun02 inactivation method as a potential therapy is far from possible. The Daun02 is unable to cross the blood brain barrier and thus need cannula guide to target subcortical structures. In addition, the length of the ß-galactosidase coding sequence associated a to cell-specific promoter often exceed 4.5 kpb, limiting to the use of lentiviral vectors to specifically target a desired structure. The use of viral vector in clinical therapies needs a reliable and reproductive production method allowing obtaining large amounts of safe and highly concentrated viral vectors. As lentiviral vectors originate from HIV (human immunodeficiency virus), their safety needs to be checked. They do not have to recombine and interact with other virus, notably to avoid the synthesis of an active HIV. In addition, the risk of mutational events occurring after lentiviral integration into the genome is a major concern to use these vectors in clinical therapies, as it could alter the expression of essential or proto-oncogene genes. Otherwise, in clinic, a fine regulation of the transgene expression is a crucial point to control the dose of the newly synthesized therapeutic protein and to stop the treatment if side effects occur. Ethically, although PD and LID are very disabling, patients can live for a long time with their deficits associated to treatments that can alleviate some of their debilitating symptoms. Recently, Palfi and co-workers designed a lentiviral vector-based gene therapy aiming to restore local and continuous dopamine production in patients with

advanced PD (Palfi *et al.*, 2014). Interestingly, no serious adverse events related to the study drug or surgical procedures were reported while improvement in motor behaviour was observed in all patients. Nowadays, several other clinical trials using adeno-associated viral (AAV) vectors are under investigation for PD including injections of AAV-GAD (glutamic acid decarboxylase) in the STN (Neurologix), AAV-AADC (aromatic L-amino acid decarboxylase) in the striatum (Avigen) and AAV-NTN (neurturin) in the striatum (Ceregene).

Interestingly, a new behavioural-compatible AAV vector-based method allows modulating the neuronal electrical activity without cannula guide implantation: the designer receptors exclusively activated by designer drugs (DREADD) (Farrell *et al.*, 2013; Ferguson *et al.*, 2011; Ferguson *et al.*, 2013; Nair *et al.*, 2013). DREADDs are engineered G-protein coupled receptors (Armbruster *et al.*, 2007), which are activated by an inert drug-like small molecule such as the clozapine-N-oxide (CNO) inducing an activation (DREADD hM3Dq) or an inhibition (DREADD hM4Di) of the neuronal firing (Wulff and Arenkiel, 2012). However this method has only been used in rodents and need to be validated in a more complex animal model such as the non-human primate as we previously did with the Daun02 inactivation method.

A previous study on drug-abuse demonstrated that the specific inactivation of LHb through DBS decreases cocaine-seeking behaviour (Friedman *et al.*, 2010). As discussed in the introductive review, DBS in the basal ganglia (STN or GPi) is a powerful strategy to alleviate LID and PD symptoms in clinic. Therefore, as we demonstrated that structures outside of the basal ganglia could functionally be involved in LID, we could target them by DBS. While the deepness and the small size of the BST is a disadvantage to target this nucleus, the LHb, which is bigger and an upper subcortical structure, seems to be a compatible target.

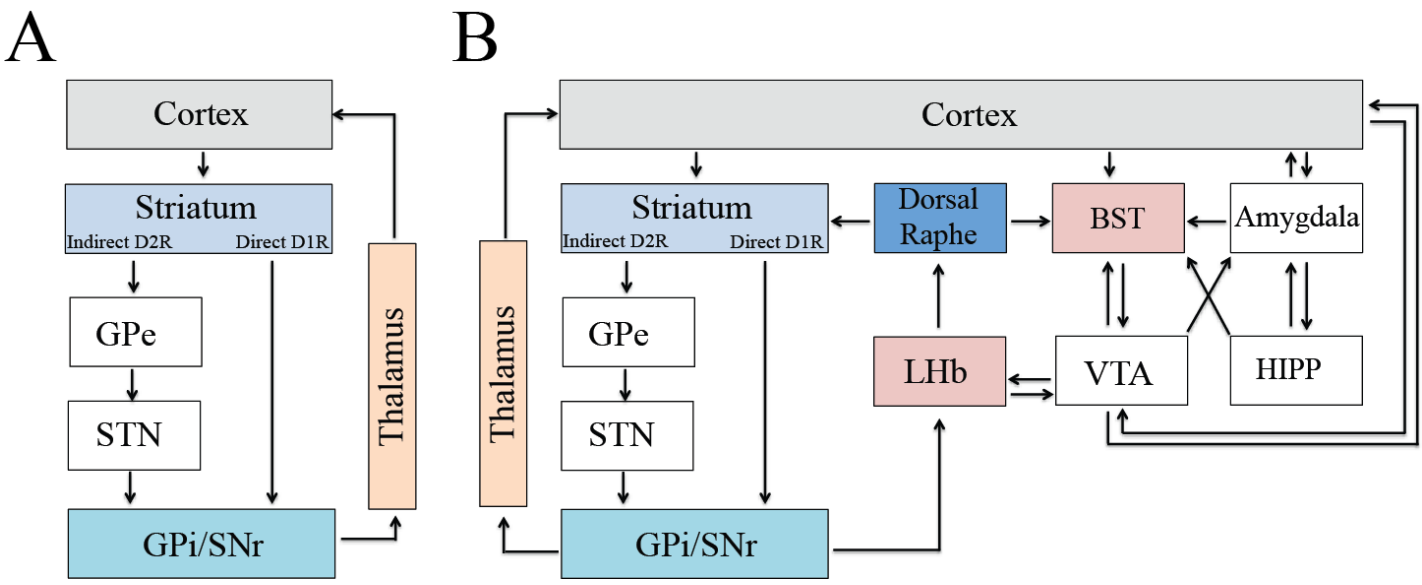

Figure 7. Concluding illustration of brain nuclei altered by a chronic L-Dopa treatment.

A– Schematic representation of the basal ganglia structures classically studied in LID pathophysiology **B–** Schematic representation including the projections and the structures outside of the basal ganglia putatively involved in LID pathophysiology.

6. Concluding remarks

In my PhD, our work shed light on the global alterations induced by a chronic L-Dopa treatment in PD. We demonstrated that dopamine replacement therapy does not only impact the structures classically studied in LID pathophysiology **(Figure 7A)** but the whole brain **(Figure 7B)** through molecular modifications leading to alterations in plasticity engaging, notably, the IEGs. Interestingly, these modifications involve motor, cognitive and limbic circuits both inside **(Figure 7A)** and outside the basal ganglia **(Figure 7B)**. Therefore we propose that the functional impact of IEG-expressing neurons upon LID severity underlies the neuronal mechanisms of LID pathophysiology involving motor complications that could be enhanced directly or indirectly by affective, motivational or cognitive alterations induced by a chronic L-Dopa treatment.

Taking in consideration the mechanisms involved in both motor and non-motor alterations could provide a more integrative insight of LID pathophysiology. Indeed, behaviours are not only "motor-related" but also include a motivational component: "I want to take a glass of water because I'm thirsty". Therefore, the pathological-related modifications induced by a chronic L-Dopa treatment in structures outside of the basal ganglia should be studied in more details to provide a better understanding of the multifactorial components impacting the motor complications in LID pathophysiology.

Supplementary publication

1. Publication 1: PSD-95 expression controls L-Dopa dyskinesia

Matthieu Bastide and Erwan Bezard

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PSD-95, une nouvelle protéine contrôlant les dyskinésies induites par la L-DOPA

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> La maladie de Parkinson est une maladie neurodégénérative caractérisée par une perte progressive de plusieurs populations neuronales, incluant notamment les neurones dopaminergiques de la substance noire *pars compacta*. Sur le plan clinique, cette maladie se traduit par trois symptômes moteurs majeurs : l'akinésie, la rigidité articulaire et les tremblements. L'objectif des traitements actuels est de pallier la déficience en dopamine, soit par l'utilisation d'agonistes dopaminergiques, soit par l'administration de Levodopa (L-Dopa ou L-3,4-dihydroxyphénylalanine), un précurseur direct de la dopamine. Bien qu'efficace pendant quelques années, la L-Dopa induit systématiquement des complications motrices se traduisant par des mouvements anormaux involontaires, appelés dyskinésies [1, 2].

Les dyskinésies induites par la L-Dopa dans le traitement de la maladie de Parkinson

À l'heure actuelle, il n'existe pas de traitement efficace permettant de lutter contre les dyskinésies. Néanmoins, plusieurs stratégies sont utilisées afin de soulager les patients. Tout d'abord, afin de retarder le plus longtemps possible la prise de L-Dopa, des agonistes dopaminergiques peuvent être administrés au stade initial de la maladie, seuls ou en combinaison avec la L-Dopa. Il est également possible de stabiliser les taux de dopamine dans le cerveau en administrant des inhibiteurs des enzymes de dégradation de la dopamine, comme la catéchol-O-méthyl-transférase (tolcapone, entacapone) ou la monoamine oxydase B (sélégiline, rasagiline). En ce qui concerne les traitements pharmacologiques anti-dyskinétiques, seule l'amantadine est prescrite. Cependant, son utilisation reste limitée par son efficacité et des effets secondaires indésirables. Une intervention neurochirurgicale est également possible. Son objectif est de permettre une stimulation cérébrale profonde, soit du noyau sous-thalamique, soit du *globus pallidus* interne. Cette approche permet, non seulement de diminuer les dyskinésies, mais également de réduire de moitié les doses de L-Dopa administrées aux patients.

Modifications de l'expression neuronale de protéines de signalisation au cours des dyskinésies

Au cours de ces dernières années, l'évolution des connaissances sur les dyskinésies a fait émerger de nouveaux concepts. La dégénérescence de la voie nigro-striée, caractéristique de la maladie de Parkinson, induit des dysfonctionnements dans la signalisation des récepteurs de la dopamine comme du glutamate. Sur le plan moléculaire, la distribution subcellulaire et les interactions fonctionnelles des récepteurs de la dopamine et du glutamate semblent jouer un rôle central dans la maladie de Parkinson, mais également dans le développement des dyskinésies. Ainsi, notre équipe a précédemment mis en évidence une augmentation du nombre des récepteurs de la dopamine de type D1 à la membrane plasmique des neurones épineux du striatum (structure cible de la dopamine) lors des dyskinésies [3, 4], alors qu'ils devraient être normalement internalisés après leur stimulation par la dopamine. Il a également été montré que les dyskinésies entraînaient une diminution de l'expression neuronale de deux protéines, la *G protein-coupled receptor kinase 6* (GRK6) et l'arrestine 2, impliquées dans le mécanisme de désensibilisation homologue [5] qui conduit à l'internalisation des récepteurs suite à leur stimulation. Or, la surexpression de GRK6 dans le striatum de rongeurs et de primates développant la maladie de Parkinson entraîne une diminution des dyskinésies [6]. Les récepteurs du glutamate sont également impliqués : des études ont montré une augmentation du nombre de récepteurs glutamatergiques de type NMDA (N-méthyl-D-aspartate) [7, 8] et AMPA (α-amino-3-hydroxy-5-méthylisoazol-4-propionate) [9] à la membrane plasmique de neurones striataux dans un modèle de primate développant la maladie de Parkinson avec des dyskinésies.

Rôle de PSD-95 dans l'internalisation du récepteur D1 au cours des dyskinésies

Les augmentations concomitantes du nombre des récepteurs D1 et du glutamate suggèrent une activation anormale de protéines impliquées dans la signalisation en aval de ces récepteurs. La protéine *postsynaptic density* 95 (PSD-95) participe à la signalisation glutamatergique et, ainsi, à la régulation de l'activité synaptique. Or, des éléments indiquent que PSD-95 pourrait également interagir avec le récepteur D1 [10] et, ainsi, réguler sa distribution membranaire et ses fonctions [11, 12].

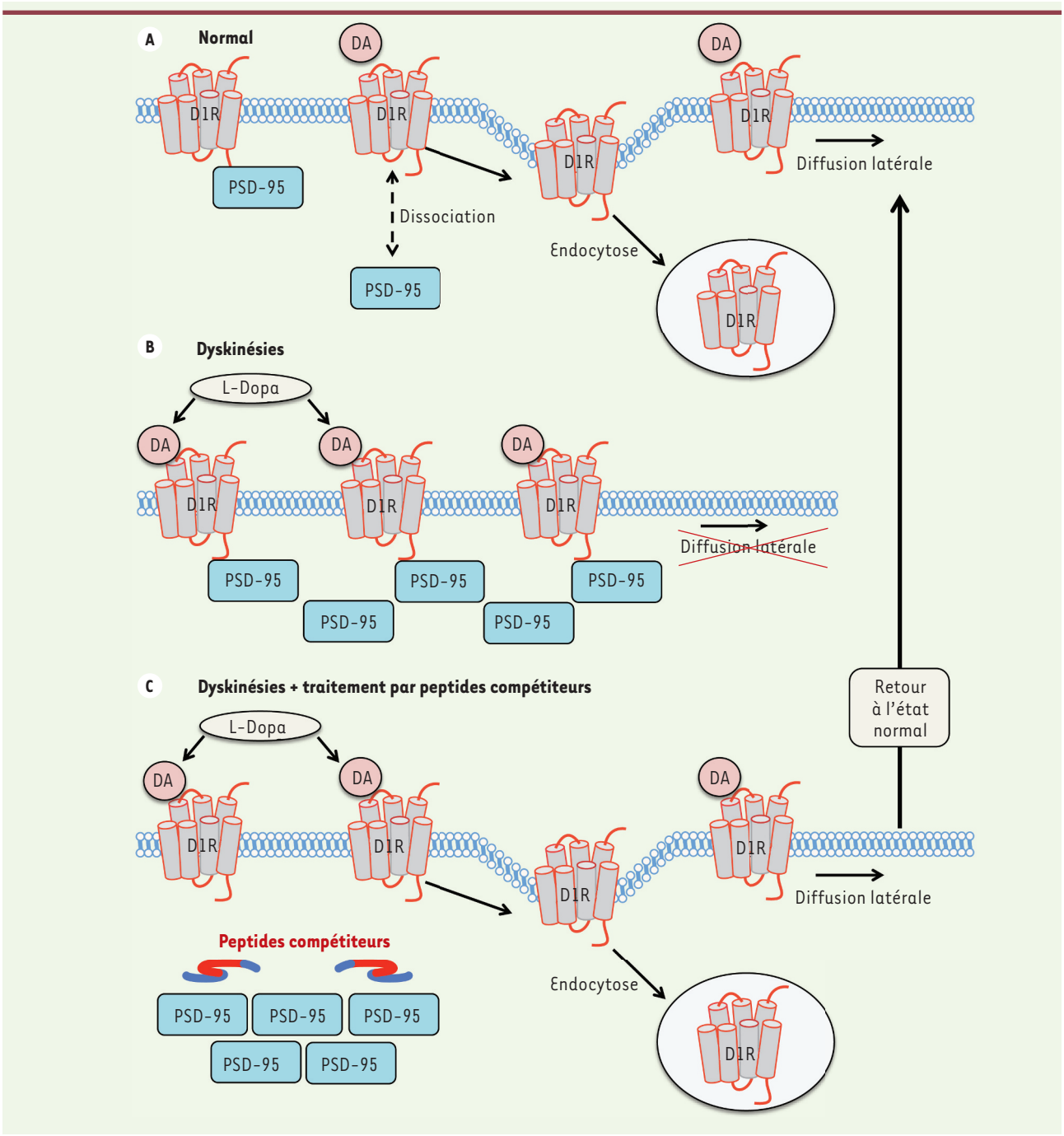

Figure 1. ***PSD-95 contrôle les dyskinésies induites par la L-Dopa. A.*** En situation normale, la stimulation des récepteurs dopaminergiques par la dopamine (DA) et, plus particulièrement celle du récepteur D1 (D1R), entraîne une diffusion latérale de ce dernier dans le plan de la membrane plasmique des neurones striataux. Par la suite, soit D1R reste associé à la membrane loin du lieu de stimulation, soit il est internalisé et recyclé afin d'éviter une stimulation continue. Cette diffusion, dite latérale, implique des protéines intervenant dans la signalisation de ces récepteurs, comme PSD-95 qui, en situation normale, peut se dissocier du récepteur D1 après sa stimulation et le « libérer ». ***B.*** Le traitement chronique par la L-Dopa entraîne le développement de mouvements anormaux involontaires appelés dyskinésies, conséquence d'une augmentation massive des taux de dopamine dans le cerveau. Lorsque ces dysfonctionnements sont manifestes, PSD-95 est surexprimée. L'accumulation de PSD-95 entraîne une immobilisation du récepteur D1 à la membrane plasmique. Il ne peut donc ni diffuser latéralement, ni être internalisé. ***C.*** L'inhibition de l'interaction directe entre PSD-95 et le récepteur D1, *via* des peptides compétiteurs injectés dans le striatum, permet de diminuer considérablement les dyskinésies. Sur le plan moléculaire, ce phénomène se traduit par une modification de la distribution membranaire du récepteur D1, qui peut alors diffuser latéralement ou être internalisé. Ainsi, les animaux traités par ces peptides peuvent bénéficier pleinement du traitement à la L-Dopa sans dyskinésies sévères.

En effet, il a été montré que les niveaux de PSD-95 étaient considérablement augmentés chez un modèle rongeur de la maladie de Parkinson, dyskinétique [13]. Nous avons alors voulu corriger la surexpression de PSD-95 afin de restaurer une signalisation normale [14]. L'étude comportementale secondaire à l'inhibition de PSD-95 a d'abord été réalisée chez le rongeur, dans un modèle de maladie de Parkinson : celui du rat unilatéralement déplété en dopamine par l'injection intracérébrale de 6-hydroxydopamine, puis traité par L-Dopa de façon chronique, ce qui induit des dyskinésies. Puis, un ARN interférent inhibant spécifiquement l'expression de PSD-95 a été sélectionné, et cloné dans un vecteur lentiviral injecté dans le striatum. Cette thérapie génique a permis de réduire considérablement les dyskinésies dans ce modèle. Des expériences de coimmunoprécipitation ont permis de vérifier l'implication du récepteur D1 dans ce phénomène, en confirmant l'interaction entre PSD-95 et le récepteur D1, et en identifiant le domaine d'interaction avec PSD95 au niveau du récepteur D1. Un peptide correspondant à cette séquence et permettant ainsi de rompre l'interaction entre le récepteur D1 et PSD-95 par compétition a été synthétisé puis injecté dans le striatum. Comme le faisait l'ARN interférent anti-PSD-95, le peptide a permis de diminuer les dyskinésies au pic de L-Dopa, ce qui démontre le rôle clef de l'interaction entre le récepteur D1 et PSD-95 dans les dyskinésies chez ce modèle rongeur. Afin de se placer dans un contexte de recherche translationnelle, nous avons répété ces travaux chez le singe macaque intoxiqué au 1-méthyl-4-phényl-1,2,3,6-tétrahydropyridine (MPTP), une autre molécule neurotoxique spécifique des neurones dopaminergiques, et considéré comme le modèle expérimental de référence de la maladie de Parkinson. Dans ce modèle, l'inhibition de PSD-95 dans le striatum par ARN interférence permet également de diminuer la sévérité des dyskinésies de façon remarquable, confirmant le rôle de PSD-95 dans les dyskinésies chez le singe. Enfin, pour approfondir le mécanisme d'action de PSD-95 sur le récepteur D1, des expériences d'imagerie de neurones striataux en culture ont été réalisées. Elles montrent que l'inhibition de PSD-95 et, plus particulièrement, l'absence d'interaction entre le récepteur D1 et PSD-95 perturbe la localisation du récepteur D1 *via* une augmentation de sa diffusion latérale *(Figure 1)*. Cette dernière peut alors être suivie d'une augmentation de l'internalisation du récepteur et, donc, d'une diminution de son expression à la membrane plasmique des neurones, permettant ainsi de restaurer la désensibilisation homologue du récepteur D1.

Conclusion

L'ensemble de ces données soulignent l'importance du rôle fonctionnel de PSD-95 dans le développement des dyskinésies et valident son intérêt thérapeutique. En effet, nous avons démontré que la surexpression pathologique de PSD-95 immobilise le récepteur D1 de la membrane plasmique des neurones, réduisant ainsi sa mobilité à l'intérieur et à l'extérieur des synapses. Or, si cette mobilité est facilitée, soit en inhibant l'expression de PSD-95, soit en empêchant son interaction directe avec le récepteur D1, il en résulte une diminution drastique des dyskinésies. Par conséquent, sachant qu'une stratégie de thérapie génique utilisant des vecteurs viraux n'est pas privilégiée, l'utilisation de peptides compétiteurs, comme celui utilisé dans cette étude, pourrait s'avérer efficace dans le traitement des dyskinésies. ◊

PSD-95 expression controls L-Dopa dyskinesia

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